ERRATA

Page	Line	Text Reads	Text Should Read
viii	8	Exo^{+} *ade* Nod^{-}	Exo^{+} Ade^{-} Nod^{-}
3	9	*parasponia*	*Parasponia*
6	8	*Medicargo*	*Medicago*
	20	senesce	senescence
10	28	oxygen-buffering	oxygen-transporting
13	27	resistance to fungus	resistance to the fungus
16	7	*Rhizobiacease*	*Rhizobiaceae*
17	28	lead	led
18	1	Nodulation	nodulation
	2	*nifH* and *nifD*	*nifHDK*
	17	Henneck	Hennecke
Table 1.1		*R.Trifolii*	*R.trifolii*
26	24	petri	Petri
29	14	CuSO:$_4$	$CuSO_4$
35	25	transmissibility	transferable
37	10	Mnso$_4$	$MnSO_4$
40	16,17	hypochloride	hypochlorite
47	27	*Sandard saline citrate*	Standard saline citrate
	29	Tris-sodium citrate	tri-sodium citrate, HCl
Table 3.1		lesss	less
Table 3.2		musoid, samll, callii	mucoid, small, calli
Table 3.5		moicoid-defective	mucoid-defective
		clevage	cleavage
legend		Tn5 has one cleavage site of *Eco*RI or *Cla*I	Tn5 has no *Eco*RI or *Cla*I sites
Table 4.4		Fesh weight	Fresh weight
74	6	Gallbraith	Galbraith
75	4	question about whether	question whether
79	14	lamellea	lamallae
84	19	Baner	Bauer
98	13	*ade*$^{-}$	Ade^{-}
99	5	*juponicum*	*japonicum*
119	20	to together with	together with
131	6	*parasponia*	*Parasponia*
132	10	Hohnston	Johnston
133	10	*Glycinemax*	*Glycine max*
136	1	Sruface	Surface
139	16	*Audira*	*Andira*
143	1	conserved	Conserved
146	1	Puler	Puhler
147	3	genet	Genet.
150	4	some	Some
	17	Biotichnology	Biotechnology
152	1	Ausurel	Ausubel
154	8	legumes	Legumes
156	6	Bacterial	Bacteriol
	14	Niehans	Niehaus
	17	Symbioric	Symbiotic
159	4	vincent	Vincent
	7	Enr.	Env.
	13	Heigelberg	Heidelberg
	23	Namitoba	Manitoba
160	1	Djorjevic	Djordjevic
	11	root-fair	root-hair
161	1	watson	Watson
	19	priefer	Priefer
162	6	cateroid	bacteroid
163	16	Shesis	thesis
	21	Plants	plants
165	19	Oreme-Johnson	Orme-Johnson

STUDIES ON THE ROLE OF EXOPOLYSACCHARIDES IN *RHIZOBIUM* INFECTION OF PLANTS

Thesis submitted for the degree of

Doctor of Philosophy

at the Australian National University

by

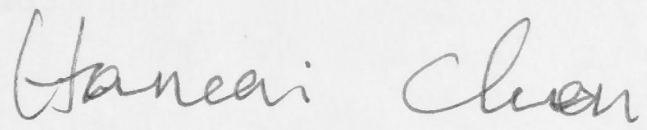
HANCAI CHEN

September, 1987

DECLARATION

The research described in this thesis is my own work, except where acknowledgement is made, and has not been submitted for any other degree.

H. Chen



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ABBREVIATIONS

Ap	Ampicillin
Cb	Carbencillin
Cm	Chloramphenicol
Km	Kanamycin
Rif	Rifampicin
Sm	Spectinomycin
Sp	Streptomycin
Tc	Tetracycline
Tris	Tris-(hydroxymethyl)aminomethane
kb	Kilobase pairs
EPS	Exopolysaccharide
CPS	Capsular polysaccharide
LPS	Lipopolysaccharide
Muc^+	Mucoid
Muc^-	Non-mucoid
Exo^+	Normal exopolysaccharide production
Exo^-	Exopolysaccharide-deficient
exo	Gene involved in EPS synthesis

Nod^+	Nodulation-proficient
Nod^-	Non-nodulating
Sym	Symbiotic
nod	Nodulation gene
hsn	Host specific nodulation
Fix^+	Effective or nitrogen-fixing symbiosis
Fix^-	Ineffective or non-nitrogen-fixing symbiosis
nif	Nitrogenase gene
hsf	Host specific nitrogen fixation
*Bam*HI	Restriction enzyme from *Bacillus amyloliquefaciens* H
*Eco*RI	" " " *Escherichia coli* BS5
*Hind*III	" " " *Haemophilus influenzae* Rd
*Xho*I	" " " *Xanthomonas holcicola*
*Cla*I	" " " *Caryophanon latum* L
R′	R-prime plasmid
r	Resistant
s	Sensitive
OPD	Osmiophilic Droplets
ANU	Australian National University
USDA	United States Department of Agriculture

ABSTRACT

Strain NGR234 is a broad host range *Rhizobium* which can effectively nodulate a broad spectrum of legumes. Ninety mutants with altered exopolysaccharide production were isolated after strain NGR234 was subjected to transposon Tn*5* mutagenesis. These mutants were classified, on the basis of their physiological properties, into nine groups. Their symbiotic properties were tested on four legumes (*Macroptilium atropurpureum, Desmodium intortum, Desmodium uncinatum* and *Lablab purpureus*) which form spherical (determinate) nodules and on the legume *Leucaena leucocephala* which forms cylindrical (indeterminate) nodules. On these plants strain NGR234 forms nitrogen-fixing nodules (Nod^+Fix^+). The results from the testing of the various mucoid-defective mutants on these plants show that it is possible to alter the synthesis of the surface polysaccharides of strain NGR234 and produce a narrower host-range Nod^+ Fix^+ *Rhizobium* strain.

Many transposon Tn*5*-induced exopolysaccharide mutants of strain NGR234 which were non-mucoid (Muc^-) did not produce normal nodules on *Leucaena* plants. Instead, these Muc^- (unconditional rough) mutants caused root hair curling and formed callus-like structures. When strain ANU265 (a Muc^+, Sym plasmid-cured derivative of strain NGR234) was coinoculated with some Muc^- callus-forming strains, normal nitrogen-fixing nodules (Nod^+Fix^+) were formed. Both Muc^- and Muc^+ strains were present in the nitrogen-fixing nodules and no genetic exchange was detected between the strains. However, some Muc^-

strains, when coinoculated with strain ANU265, produced normal-sized nodules (instead of callus-like structures) that were unable to fix nitrogen. Examination by light microscopy of the callus-like structures produced by the Muc$^-$ (callus-forming) mutants on *Leucaena* plants suggested that they are deformed nodules resulting from early inhibition of the normal infection process. Experiments with a specifically constructed transconjugant strain which produces varying amounts of exopolysaccharide (EPS) support the conclusion that EPS is essential for correction of the nodulation behaviour of Muc$^-$ mutants.

A Group 9 mutant strain ANU2895 induced nitrogen-fixing nodules on the non-legume *Parasponia andersonii* while the parent strain NGR234 formed ineffective nodules (Fix$^-$) on *Parasponia*. The Fix$^+$ *Parasponia* nodules formed by strain ANU2895 were similar to the Fix$^+$ nodules induced by a *Parasponia Bradyrhizobium* strain CP279 in both morphology and nitrogen fixation capacity although the effective nodulation frequency of strain ANU2895 was lower than that of strain CP279. This newly created *Parasponia Rhizobium* strain contained a single Tn*5* insertion.

The infection of the tropical legume *Leucaena leucocephala* by strain NGR234 was studied using both light and electron microscopy. *Rhizobium* infection of *Leucaena* was shown to be via infection threads formed in root hairs. Electron microscopic studies revealed both inter- and intra-cellular infection threads in nitrogen-fixing nodules. Root hair deformation and infection was mostly on lateral roots but occasionally found on taproots. The lateral roots appeared on plants three days after two and a half day-old seedlings were transferred to the growth medium in the assay vessel. Root hair curling or branching was first observed five days after *Rhizobium*

inoculation. Infection threads were seen on each plant between ten to eighteen days after inoculation. Nodules were visible on plants twelve days after inoculation and most of them were located on lateral roots. The addition of exopolysaccharide from strain ANU280 or the oligosaccharide repeat-unit from the EPS did not cause any detectable changes to the root hairs.

Several transposon Tn*5*-induced mutants of strain NGR234 which did not produce exopolysaccharides (Exo^- phenotype, Group 2 mutants) were unable to induce nitrogen-fixing nodules on *Leucaena* and siratro plants. However, the abilities of three Group 2 Exo^- mutants to induce nitrogen-fixing nodules on *Leucaena* and siratro were restored by coinoculation with purified EPS from the parent strain, or the oligosaccharide from the EPS. In addition, the Exo^- mutants were able to form small ineffective nodules rather than calli on *Leucaena* in the presence of added EPS from a Group 8 mutant ANU2858. The EPS from the parent strain was able to enhance the nodulation ability of mutant ANU2858 to induce larger but still ineffective nodules on siratro. The EPS from a Group 9 mutant ANU2861 had the similar effect as the EPS from the parent strain. The defective symbiotic phenotype of mutant ANU2861 was not altered by the addition of the EPS from the parent strain.

To initiate the fine structure genetic analysis of the various Exo^- mutants, R-prime plasmids were constructed from strain NGR234. These were shown to contain overlapping genomic DNA segments which encoded genes involved in biosynthesis of exopolysaccharides. These R-primes were isolated in intergeneric matings between a Tn*5*-induced EPS-deficient mutant of strain NGR234, strain ANU2811 and an *E.coli* recipient, by selecting for the transfer of the kanamycin resistant

marker of Tn*5*. A physical analysis of these R-primes showed that the *Rhizobium* DNA regions inserted, ranged from 59-84kb in size and were homologous to the corresponding regions where Tn*5* was located in the original mutant strain. It was demonstrated that the presence of the R-prime carrying the mutant ANU2811 allele (R'2811-9) had a dominant effect on the regulation of EPS biosynthesis of the wild-type strain NGR234 and two heterologous strains of fast-growing *R. japonicum* and *R. meliloti*. In addition, two Exo^{+} *ade*$^{-}$ Nod^{-} mutants (ANU2861 and ANU2866) carrying R'2811-9, had Exo^{-} phenotypes and were able now to ineffectively nodulate siratro and *Desmodium* spp. although still *ade*$^{-}$. DNA hybridization showed that the R-prime did not carry the 2861-allele. No instability of the R-prime plasmids was observed in the parent strain ANU280. The dominance phenotype and stability of the Tc^{r} Km^{r} R-primes enabled the isolation of a set of Tc^{r} Km^{s} R-prime plasmids carrying wild-type sequences of strain NGR234. These Tc^{r} Km^{s} R-primes were isolated in intergeneric matings between *Rhizobium* and *E.coli* with selection for the loss of the dominant phenotype and the kanamycin-resistance marker of transposon Tn*5*. A physical analysis of these Tc^{r} Km^{s} R-primes demonstrated that they carried wild-type sequences of the parent strain NGR234 and had lost the original Tn*5* insertion due to homologous recombination.

R-prime plasmids which carry overlapping segments of wild-type DNA from strain NGR234, were able to correct the Exo^{-} phenotype for 28 of the 30 Group 2 Exo^{-} mutants of strain NGR234. Complementation of these Group 2 Exo^{-} mutants with these R-primes also restored their symbiotic abilities of effective nodulation. *In vivo* recombination between the wild-type alleles located on the R-prime and the corresponding mutated allele on the genome, was used to generate a new

family of R-primes which carried mutations in the genes involved in exopolysaccharide synthesis (*exo*). The 30 Group 2 Exo$^-$ mutants were classified into seven distinct genetic-groups based upon complementation and physical mapping. Five of the seven *exo* loci are clustered on a 15kb region of DNA. Mutations at two of the five *exo* loci are dominant when the mutation was plasmid-located while a mutation at a third locus was *cis* dominant to two other *exo* loci. At least five genes involved in the synthesis of acidic exopolysaccharide have been identified in strain NGR234.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 CLASSIFICATION OF THE *RHIZOBIUM*-LEGUME SYMBIOSIS

The bacterial genus *Rhizobium* is a taxonomic grouping based on the ability of the bacterium to form a nitrogen-fixing symbiosis with plants of the family Leguminosae. This family of plants contains nearly 20,000 members. To date, only 20% of legume species have been tested for nodulation ability and it has been shown that not all species or genera are capable of nodulation (Allen and Allen, 1981).

Less than a decade after the discovery of *Rhizobium* as the microsymbiont (Beijerink, 1888), it has been widely demonstrated by plant nodulation tests that certain strains of *Rhizobium* possessed the ability to nodulate specific legume species. From these plant nodulation analyses developed the "cross-inoculation group" concept, summarized by Duggar in 1897 (Cited in Fred *et. al.*, 1932):

"As a rule, a *Rhizobium* isolate which induces the growth of nodules on one legume is unable to produce nodules on plants belonging to other genera".

This concept has been applied to many easily accessible legumes over the years and has lead to the classification of the *Rhizobium* genus into species. For example, a strain of bacteria isolated from a

clover (*Trifolium*) species will nodulate a number of clover species and is therefore regarded as *R. trifolii*. The major cross-inoculation groups and species of *Rhizobium* are shown in Table 1.1. An alternate classification of *Rhizobium* summarized by Norris (1956) divides the genus into three groupings based on colony morphology and growth characteristics. These three groupings include bacteria that nodulate: a) tropical legumes, b) alfalfa and c) temperate legumes. Both systems provide a convenient way of classifying *Rhizobium* isolates. In addition, Norris (1956) has presented a hypothesis which relates *Rhizobium* taxonomy to the evolutionary development of the legumes. Open-pollinated ancestral legumes were tropical in origin and were promiscuously nodulated by slow-growing, alkali-producing *Rhizobium* species (Norris, 1956). Temperate legumes, decendents of the ancestral tropical legumes, are generally characterized by self-fertilization and a preference for calcareous (alkaline) soils (Norris, 1956). The *Rhizobium* species associated with temperate legumes are fast-growing, acid-producing and nodulate very narrow ranges of host plants (Norris, 1956). Thus, the frequently studied symbiotic associations with clovers, alfalfa and peas are much more specialized than those of tropical legumes.

There are many examples of certain strains of bacteria which can cross the boundaries of this classification system to give an effective nitrogen-fixing symbiosis on a plant of another cross-inoculation group, and this has lead some authors (Wilson, 1944) to question the validity of the cross-inoculation system. However, as a general rule it holds, since the majority of strains within a cross-inoculation group will interact only poorly with plants of another group.

As more research is done on the vast number of legumes present throughout the world there are certain to be new groups and reordering of others, especially the cowpea miscellany. This group contains a large number of rhizobia which have a broad host-range and can usually infect a variety of tropical legumes. In fact, the slow-growing rhizobia which comprise most of the cowpea group of bacteria have been classified recently in a separate group known as the *Bradyrhizobium* (Jordan, 1984). *Bradyrhizobium* strains have also been identified which nodulate the non-legume tree *parasponia* (Ulmaceae) (Trinick, 1973; 1980a). Other than this unusually broadened host-range, these slow-growing *Rhizobium* strains resemble other *Bradyrhizobium* strains in most respects (Trinick and Galbraith, 1980).

1.2 INFECTION OF LEGUMES BY *RHIZOBIUM*

A successful invasion of a legume plant by *Rhizobium* that leads to an effective nitrogen-fixing symbiotic relationship is an exceedingly complex process which is only now starting to be understood genetically and biochemically. A number of comprehensive reviews have been published (Dart, 1977; Sprent, 1979; Vincent, 1980; Bauer, 1981) which describe this complex interaction between the bacterium and the plant.

Microscopic analysis of the establishment of a nodule (mainly temperate legume nodulation) has revealed morphologically distinct developmental steps. These steps, defined by Vincent (1980) and Rolfe *et al.* (1981), are presented diagrammatically in Figure 1.1. The binding of *Rhizobium* to the root hairs of the legume host can lead to their distortion such that they take on a bent or curled shape (see

Bauer, 1981). Root tissue is then invaded by *Rhizobium* through the formation of an infection thread in the curled root hair. This is formed by fusion of both smooth and coated vesicles derived from Golgi bodies with plasma membranes at the site of infection (Robertson and Lyttleton, 1982). The infection thread elongates, passing through the root hair cell and into the root cortex. This process is accompanied by the initiation of cell division in inner cortical cells ahead of the advancing infection thread giving rise to the development of a meristematic zone. The infection thread continues to grow and branch into the dividing cortical cell region. Plant cell growth from this region eventually forms the highly differentiated structure referred to as a nodule. As this occurs the plant vascular system develops to encompass this zone of rapid development (Dart, 1977). *Rhizobium* bacteria are released from the infection thread by the disintegration of the walls of this structure. The bacteria become embedded in a mucopolysaccharide matrix and are surrounded by a plant derived membrane, the peribacteroid membrane (Robertson *et. al.*, 1978). *Rhizobia* subsequently differentiate often into large pleiomorphic forms termed bacteroids (Libbenga and Bogers, 1974; Newcomb, 1980). Nitrogen fixation (*nif*) genes are expressed and the nitrogen-fixing phenotype is elaborated only in the bacteroid form. Fixed nitrogen is not metabolized but is exported (Bergersen and Turner, 1967) and assimilated by plant-encoded enzymes (see Kahn *et. al.*, 1985).

Although work on infection has concentrated on root hair curling and consequent infection thread synthesis, other kinds of infection are known. In tropical legumes, such as peanut (*Arachis hypogaea*) and stylo (e.g. *Stylosanthes hamata*), infection threads are not found in the nodules (see Dart 1977). The only publications on infection of

such legumes are those of Chandler (1978) and Chandler *et al.* (1982) which describe the infections of peanut and stylo respectively. Root nodules of these legumes occur only at lateral root junctions and result from direct invasion by rhizobia through spaces between epidermal cells (crack entry). Invasion of the host cortical cells occurs through structurally altered cell walls. The bacteria reach the site of nodule initiation in the root cortex by progressive collapse of the initially invaded cells, which are compressed by neighbouring cells to form intercellular thread-like infection zones (the author suggests that this resembles the defence mechanisms of some plants to invasion by pathogenic organisms, citing Tarr (1972)). The bacteria multiply in the invaded cells of the nodule initial which divide repeatedly to form the nodule. Bacteroids form only when the host cells cease to divide. Although there are minor differences between peanut and stylo infections (for example origin of nodule initials) the basic principles described by Chandler are the same.

Another kind of infection is that of stem nodulation, known to occur in *Neptunea oleracea, Aeschynomene indica,* and *Sesbania rostrata.* Duhoux (1984) describes stem nodules of *S. rostrata* having a large central mass of infected cells. Stem "mammillae" (points of quiescent adventitious roots) are regularly arranged in vertical files along the stem and develop into nodules when they are infected by a specific *Rhizobium.* Each nodule arises from the development of an infected region of the incipient root cortex. The infection in *S. rostrata* has been shown to proceed in four sequential stages (Duhoux, 1984). According to Duhoux, some of them have never been shown to occur in other legumes: (i) bacterial penetration takes place in degenerated (dead) cortical cells; (ii) proliferation of the bacteria

occurs in the intercellular cavities and initiates a meristematic nodule; (iii) protrusion of infection threads at first occurs intercellularly and then intracellularly from the cavities; (iv) finally there is an intracellular release of rhizobia by an endocytotic process.

The nodule structures which develop during the formation of the *Rhizobium*-legume symbiosis comprise two types (Sprent, 1979). As a general rule, temperate legumes (e.g. *Pisum sativum, Medicargo sativa* and *Trifolium* species) develop "indeterminate" nodules upon infection by fast-growing *Rhizobium* strains. Tropical legumes (e.g. the *Glycine, Vigna, Lupinus, Macroptilium* and *Arachis* genera) develop "determinate" nodules in symbiosis involving *Bradyrhizobium* species. In the indeterminate nodules, meristematic activity continues, forming new regions of bacteroid-containing plant tissue at the end of the nodule while older areas senesce. The continued outgrowth of the nodule in this manner results in a cylindrical shape.

In contrast to indeterminate nodules, plant cell divisions in the determinate nodule terminate at an early stage. Consequently, mature determinate nodules of this type do not show the outward zone of meristematic activity seen in indeterminate nodules and senesce occurs through the whole nodule rather than in a defined zone (see Sprent, 1979; Rolfe and Shine, 1984). These nodules appear spherical in shape.

The stages where host specific interactions occur during the developmental sequence of nodule formation are just beginning to be determined (Djordjevic *et. al.*, 1987). Root hair curling and deformation do not appear to be strictly host specific although there appears to be a degree of specificity between plants of different sub-

families (Yao and Vincent, 1969; Djordjevic *et. al.*, 1986). Actual root hair curling and deformation may not be strictly necessary for nodulation since many plants which nodulate form no curled root hairs and alternate infection strategies like those observed on peanut and stylo take place. Root hair curling is probably an adjunct to infection, acting to increase the probability of trapping *Rhizobium* cells in a site favorable for penetration of the plant (Verma and Nadler, 1984). This penetration step may be host specific although little is known about this stage in nodule development. Heterologous rhizobia which are able to curl the root hairs of plants of another cross-inoculation group are reportedly unable to form infection threads (Yao and Vincent, 1969).

Even if a bacterium can invade the root and initiate a nodule there are still host specific interactions at stages of induction of a nitrogen-fixing symbiosis. The same strains of *Rhizobium* may form effective nodules on one plant and ineffective nodules on another (Nutman, 1956). Similarly, a plant may develop effective nodules with one strain and ineffective nodules with another. Furthermore, the effective nodules vary in their efficiency of nitrogen fixation (Nutman, 1969; Caldwell and Vest, 1977). Thus, there is a continuous interaction in the compatibility of both bacteria and host during the development of effective symbiosis.

1.3 NODULATION OF *PARASPONIA*

The non-legume *Parasponia* spp., belonging to the plant family Ulmaceae, is able to produce nitrogen-fixing nodules with certain *Rhizobium* strains (Akkermans *et. al.*, 1978; Becking, 1977; Trinick,

1973). Up to now, it has a unique position as it is the only non-leguminous plant showing such a capacity.

Nodules were first observed on *Parasponia* by Ham (1909) (described by Akkermans *et. al.*, 1978) although this report was lost for many years. Trinick (1973) conclusively proved that the nodules were induced by an endosymbiotic bacterium with the characteristics of *Rhizobium*. Isolates from *Parasponia* nodules were able to infect *Parasponia* plants inducing nitrogen-fixing nodules, thereby fulfilling Koch's postulate. *Parasponia* plants cultivated aseptically do not develop nodules.

In studies of the position of *Parasponia* in the cross-inoculation group concept, Trinick and Galbraith (1980) made the startling discovery that some of the *Parasponia* bacteria could nodulate a number of legumes from the cowpea group, with varying degrees of effectiveness. Similarly, "classic" cowpea *Rhizobium* strains, such as CB756 (a widely used commercial inoculum strain) could nodulate *Parasponia*. The endosymbiont of *Parasponia* was therefore considered a *Rhizobium* species. Although most *Parasponia Rhizobium* strains were not highly effective in symbiosis with cowpea plants one strain (CP283) was able to effectively nodulate a wide range of cowpea plants.

Parasponia can also be nodulated by slow-growing *B. japonicum* and *R. loti* strains as well as numerous fast-growing rhizobia from *Leucaena* and *Mimosa*. On the basis of this evidence, Trinick assigned the *Parasponia-Rhizobium* symbiosis to the cowpea miscellany. This does not mean that all cowpea rhizobia will nodulate *Parasponia*. The reverse is also true since a number of *Parasponia Rhizobium* strains have a restricted host-range, seeming to be specialised for *Parasponia*.

Structural studies of mature nodules of *P. rugosa* (Trinick and Galbraith, 1976), *P. andersonii* (Trinick, 1979), and *P. parviflora* (Becking, 1979) indicate that the root nodules resemble more closely the actinorhizal type induced by the actinomycete *Frankia* on various non-legumes (Bond, 1974) than those typically formed on legumes in association with *Rhizobium* (Dart, 1977). Mature *Parasponia* root nodules are coralloid in shape and consist of multiple, branched lobes. Like actinorhizal nodules, each nodule lobe has an apical meristem and a single central vascular bundle surrounded by the nodule cortex which includes the zones of infected cells. The infection zone consists of host cells filled with thin-walled infection threads containing bacteria (Trinick and Galbraith, 1976; Trinick, 1979). Bacteria are not released from infection threads and packaged into independent "sacs" of bacteria as found in most legume symbiosis (Dart, 1977). However, the retention of bacteria within threads in host cells also has been reported for the *Rhizobium* symbiosis with tree legumes belonging to the genus *Andira* (deFaria *et. al.*, 1986).

Although the structure of mature *Parasponia* nodules has been well documented (Trinick, 1979; Lancelle and Torrey, 1984) the early infection events were unknown until Lancelle and Torrey (1984) suggested that the presence of "multicellular root hairs" with subjacent cell divisions in the outer cortex was the first sign of root nodule initiation. Unlike *Frankia*/non-legume (Berry and Torrey, 1983) and other *Bradyrhizobium*/legume (Turgeon and Bauer, 1985) infections, root hairs were not curled and there was no initiation of infection threads within root hairs. Instead, bacteria entered via intercellular spaces within a mass of dividing cells that emerged through the epidermis and infection threads were not initiated until

bacteria reached further down into the cortex (Lancelle and Torrey, 1984). The subsequent events of nodule development were similar to those observed in *Frankia* symbiosis with other non-legumes (Lancelle and Torrey, 1985; Lalonde, 1977). More recently, it has been shown that *P. andersonii* has an infection zone close behind the growing tip of the tap root (Bender *et. al.*, 1987b) which is similar to that found in legume infections by *Bradyrhizobium* (Bhuvaneswari *et. al.*, 1980; 1981). A more detailed analysis of the temporal stages in the early infection using scanning electron microscopy showed that the process could divided into a series of consecutive steps:

1, erosion of the root surface beginning within 24 hours;

2, stimulation of cell division beneath the point of erosion which was visible in 4 days;

3, the emergence of dividing cells through the epidermis in 6 days which was closely followed by;

4, the intercellular colonization of the cortex and initiation of infection threads (Bender *et. al.*, 1987b).

It has been suggested the *Parasponia-Bradyrhizobium* symbiosis is less highly evolved than that found in most legume and other non-legume symbiosis where the symbionts gain entry by the more sophisticated mechanism of intracellular infection thread formation after the induction of root hair curling (Bender *et. al.*, 1987b). Strain NGR234 is capable of forming nodules on *Parasponia* but these nodules do not initiate nitrogen fixation (Trinick and Galbraith, 1980; Bender *et. al.*, 1987a). Although *Parasponia* nodules contain no leghaemoglobin (Coventry *et. al.*, 1976) a haemoprotein of some type (Appebly *et. al.*, 1983; Landsmann *et. al.*, 1986) is present to provide the oxygen-buffering capacity necessary for nitrogen fixation.

1.4 *RHIZOBIUM*-LEGUME VS PATHOGEN-HOST

Rhizobium-legume infection and nodulation bear a striking resemblance to infection and disease induction by plant pathogenic organisms (Vance, 1983; Djordjevic *et. al.*, 1987). However, it is intriguing that no bacterial pathogens have evolved the fine precision that characterizes the interactions of *Rhizobium* with their hosts. Earlier concepts suggesting that nodule formation and symbiosis did not evolve from a primitive pathogenic interaction may need re-evaluation (Parker, 1957). However, irrespective of the final manifestation of the *Rhizobium*-legume interaction, it has many features in common with plant-parasite infection and development (Vance, 1983; Djordjevic *et. al.*, 1987). These features include:

a) Binding of the microbes to the host plant

b) Penetration into the host plant

c) Host plant response to penetration

d) Redirection of host plant metabolism

e) Morphological manifestation of the interaction, i.e. nodules and symptoms

Approaching the *Rhizobium*-legume association from a phytopathological perspective reveals the limitations of our understanding, and may offer novel approaches to further research (Vance, 1983).

If recognition in *Rhizobium* to associate with legumes preceded their ability to reduce nitrogen, due to either a defect in the bacterium or the host, then this organism was originally a pathogen depending on the host for the supply of both carbon and nitrogen as a necrotroph or biotroph. The symbiotic relationship between *Rhizobium*

and plants can thus be viewed as that of a "controlled disease" (Rolfe *et. al.*, 1982).

Rhizobium invasion of legume plants is generally mediated by a tubular structure called the infection thread. The infection thread grows inwards reaching and spreading to the cells that become meristematic as a result of, or prior to the infection, and restricts the invader to an extracytoplasmic compartment (Verma and Nadler, 1984). The *Rhizobium* is finally released from the end of the infection thread but remains enclosed in a host membrane envelope, establishing the bacterial symbiont in the host cortical cell cytoplasm (Dart, 1977; Robertson and Farnden, 1980). Thus, the two organisms remain separated from each other throughout the endosymbiotic stage, keeping this association topologically extracellular. Such a controlled invasion may be considered to be a mechanism to avoid host defense responses against the invading "pathogen". Alternatively, deposition of infection thread material in the path of invading *Rhizobium* may be regarded as a defence response or "controlled incompatible" response between the invading bacteria and infected host plant that allows the rhizobia to reach the plant milieu but prevents their access to the cytoplasm (Djordjevic *et. al.*, 1987).

Thus, a successful infection and effective nodulation of the host plant is associated with a compatible *Rhizobium*. In the case of plant symbionts (or parasites), one can predict that they must avoid or inhibit any pathogenic responses from the plant, such as the hypersensitive reaction, which would hinder their multiplication. On the other hand, the host must not use its defense mechanism against the *Rhizobium*. This could be because of the development of a

mechanism for controlled invasion and strict compartmentalization that occurs in the nodule structure. Any breakdown in this process may render *Rhizobium* pathogenic to the host. Accurately, the lack of development of an infection thread or premature release of *Rhizobium* from the infection thread (due to genetic incompatibilities or mutations in host or bacteria (Vincent, 1980)) aborts the process of nodule development (Bassett *et. al.*, 1977b) and the *Rhizobium* infection becomes pathogenic (Verma and Nadler, 1984). The isolation of various classes of *Rhizobium* mutants unable to carry out the symbiotic process has demonstrated that a number of different steps are involved in the establishment of effective nitrogen fixation (Vincent, 1980; Rolfe *et. al.*, 1981a). When mutant strains with different symbiotic defects are mixed with each other, cellular complementation may occur to produce an effective symbiosis (Rolfe and Gresshoff, 1980). These results indicate a series of *Rhizobium*-plant interactions occurring at various stages of nodule initiation and development.

Heath (1974) refers to such stages (in the context of plant pathology) as "switching points", where the outcome of the interaction determines the subsequent progress of the infection. Thus, successful infection depends upon the "correct" response at every stage, while rejection requires an "incorrect" response at only one stage. An incorrect response could be interpreted as the triggering of an active defence mechanism or the failure to induce acceptance. It can be expected that each of the switching points represents a resistant gene, especially in the work by Ellingboe (1972) who has shown that different genes for resistance to fungus *Erysiphe graminis* in wheat and barley are manifested at different

points and in different ways during the infection process; and from the macroscopic studies of Littlefield (1973), who has shown a similar situation in the resistance of flax to fungus *Melampsora lini*. The studies presented in this thesis provide evidence for such switching points in the context of the *Rhizobium*-legume symbiosis.

1.5 GENETICS OF *RHIZOBIUM*

Krasilnikov first reported (in 1941) the transfer of host range between *Rhizobium* species (cited in Beringer *et. al.*, 1980). Transformation of auxotrophic mutants and extention of nodulation host range in *R. japonicum* (Balassa, 1956) and *R. lupini* (Balassa, 1960) also has been reported. Transduction, both specialized and generalized has been observed in *R. meliloti* and *R. leguminosarum* (reviewed by Beringer *et. al.*, 1980). Conjugation was first reported in *R. lupini* (Heumann, 1968) and a circular linkage map was established by means of this naturally-occurring conjugal system. Circular linkage maps quickly developed for *R. leguminosarum* (Beringer and Hopwood, 1976; Beringer *et. al.*, 1978b), two strains of *R. meliloti* (Meade and Signer, 1977; Kondorosi *et. al.*, 1977) and *R. trifolii* (Megias *et. al.*, 1982) when chromosome-mobilising plasmids, such as R68.45, were discovered in *Pseudomonas* (Haas and Holloway, 1976; Holloway, 1979). The linkage maps indicated a similarity between *R. leguminosarum* and *R. trifolii* but these did not extend to *R. meliloti* (Kondorosi *et. al.*, 1980). Although some Fix^- mutations were mapped on the chromosome, mapping of host range genes on host chromosomes was unsuccessful (reviewed by Beringer *et. al.*, 1980). The genetic analysis of *Rhizobium*-induced symbiosis was however,

hindered by the absence of genetically well characterized mutants. In early studies, many mutants were isolated either as spontaneous mutants or after the use of mutagens or UV irradiation. Selection of auxotrophs, strains with altered colony morphologies or mutants resistance to antibiotics or antimetabolites were obtained. Such techniques are indirect in that they depend upon the correlation between the mutant phenotype seen in pure culture and defects produced in the bacteria-plant symbiosis, which can be verified only by the isolation of revertants. Furthermore, in these studies it was impossible to know if any single mutagenic event had occurred, since these mutants were isolated from among the survivors after a mutagenic treatment.

The problems associated with these mutagenesis treatments were overcome by the introduction of transposon mutagenesis (Beringer *et. al.*, 1978a).

Transposons are discrete DNA entities which are able to translocate from one location to another in DNA, in the absence of host-mediated recombination functions, at varying frequency and with varying specificity. Moreover, they are often characterized by being associated with an antibiotic resistance gene (reviewed by Kleckner, 1981). Transposons as mutagens offer the unique advantage of allowing positive selection for an insertionally inactivated gene. Whereas, point mutations, induced by chemical mutagens or UV irradiation, can in general, only be detected after screening survivors. The transposon Tn*5* encodes resistance to aminoglycoside antibiotics (kanamycin and neomycin), transposes at low frequencies with little specificity and results in polar inactivation of downstream genes of an operon (Berg *et. al.*, 1980) (reviewed by Berg and Berg, 1983).

The conjugative broad-host-range *Inc* P1 group plasmids (Datta *et. al.*, 1971) are readily transmissible to *Rhizobium* by conjugation (Datta *et. al.*, 1971; Beringer, 1974). Broad-host-range plasmids of other incompatibility groups can also be introduced into *Rhizobium*, such as the *Inc* Q plasmids (Bagdasarial *et. al.*, 1981). Bacteriophage Mu, a transposable element used in *E.coli* genetics, is unstable in the *Rhizobiacease* genus *Agrobacterium* (van Vliet *et. al.*, 1978). Phage Mu can be introduced into *Rhizobium* via the Inc P-1 conjugative plasmids but is unstable (Boucher *et. al.*, 1977; van Vliet *et. al.*, 1978).

Since plasmids containing phage Mu are unstable in *Rhizobium*, an efficient mutagenesis system has been designed in which the transposon Tn*5* is "loaded" onto the unstable RP4::Mu plasmid. This provides an effective delivery system for the transposon due to the instability of the (suicide) vector (Beringer *et. al.*, 1978a), and resulted in efficient Tn*5* mutagenesis of *R.leguminosarum*. Such a technique allows for positive selection of mutants and, when coupled with rapid plant assays (Rolfe *et. al.*, 1980a), facilitates the screening of large numbers of mutated bacteria for symbiotic defects. Due to the low DNA sequence insertional specificity (Berg and Berg, 1983) and the fact that its aminoglycoside antibiotic resistance determinants (kanamycin, neomycin and streptomycin resistance) are easily selected for in *Rhizobium*, Tn*5* has been the transposon most frequently used. Other transposons, Tn*7* (Bolton *et. al.*, 1984) and Tn*1* (Casadesus *et. al.*, 1980) for example, have unique or limited sites of insertion in the *R. meliloti* symbiotic plasmid.

Kanamycin resistance (Km^r) acts as a genetic "flag" for the mutated locus and the lack of certain restriction endonuclease sites

within Tn*5* (Jorgensen *et. al.*, 1979) allows DNA restriction fragments containing Tn*5* to be readily isolated by molecular cloning techniques. Such procedures facilitate the isolation of corresponding wild-type restriction fragments from DNA libraries (Scott *et. al.*, 1982). These fragments can be used to demonstrate that the transposon is responsible for the genetic defect of an isolated mutant strain (Scott *et. al.*, 1982), (one of the disadvantages of chemical mutagenesis) and allow direct analysis of the gene at the DNA level.

While the transposon mutagenesis system provided the method to identify specific genes involved in general metabolism and symbiosis, the study of *Rhizobium* plasmids answered questions concerning the location and arrangement of genes responsible for symbiosis. Higashi (1967) presented data suggesting that a plasmid was responsible for host range and nodulation in *R. trifolii* by conjugal transfer of *R. trifolii* host range to *R. phaseoli*. *Rhizobium* strains treated with known plasmid-curing agents such as acridine orange lose their nodulation ability (Higashi, 1967; Dunican and Cannon, 1971). Recent work (eg Zurkowski and Lorkiewicz, 1978; 1979; Nuti *et. al.*, 1979; Zurkowski, 1980; Djordjevic *et. al.*, 1982; Kondorosi *et. al.*, 1982; Djordjevic, 1983; Djordjevic *et. al.*, 1983; Kondorosi *et. al.*, 1984; Morrison, 1984; Rolfe and Shine, 1984) has shown that the symbiotic (Sym) plasmid carries not only nodulation genes, but a range of initial infection genes, host range and nitrogen fixation genes.

Mapping of *Rhizobium* plasmids on the DNA level (Prakash *et. al.*, 1981; Kondorosi *et. al.*, 1982; Pankhurst *et. al.*, 1983; Schofield *et. al.*, 1983) has lead to the general conclusion that genes involved in symbiosis are clustered on a large segment of Sym plasmids. *R.*

leguminosarum Nodulation (*nod*) genes map about 20kb from structural nitrogenase genes (*nifH* and *nifD*) and other genes involved in nitrogen fixation and nodule development also map in this symbiotic region (Ma *et. al.*, 1982; Downie *et. al.*, 1983). The analysis of the nitrogenase operon is well advanced (Ow and Ausubel, 1983; Zimmerman *et. al.*, 1983; Corbin *et. al.*, 1983; Sundaresan *et. al.*, 1983) with the discovery that a *Klebsiella* regulatory gene (*nifA*) can activate *Rhizobium nifH* promoters.

More recent studies have shown that in *R. trifolii* and *R. leguminosarum* the *nod* genes are clustered on a 10-14kb fragment of Sym plasmid DNA (Downie *et. al.*, 1985; Schofield *et. al.*, 1984). In two strains of *R. meliloti*, separate clusters of *nod* genes are located about 12kb apart on the Sym plasmid (Kondorosi *et. al.*, 1984). In other bacteria the *nod* genes are more dispersed either over the Sym plasmid (strain NGR234) (Bachem *et. al.*, 1985; 1986; Nayudu and Rolfe, 1987) or the chromosome (*Bradyrhizobium* spp.) (Lamb and Henneck, 1986; Russell *et. al.*, 1985; Scott, 1986). Interspecies complementation studies have demonstrated that four contiguous and highly homologous genes, designated *nodDABC*, are functionally interchangeable between species (Kondorosi *et. al.*, 1984; Djordjevic *et. al.*, 1985; Fisher and Long, 1985; Scott, 1986) and thus have been called the "common" *nod* genes. The other *nod* genes determine the host specific nodulation, termed *hsn* genes (Downie *et. al.*, 1985; Knight *et. al.*, 1986; Kondorosi *et. al.*, 1986; Rolfe *et. al.*, 1986). The *hsn* genes have been defined by two criteria: (a) as those genes necessary to be introduced into a "foreign" *Rhizobium* species to extend the host range ability to include plants nodulated by the donor bacterium and, (b) by nodulation defective mutants which have

altered host range phenotypes but which cannot be complemented by the introduction of corresponding (and sometimes highly homologous) genes from other *Rhizobium* species. At present, very little is known about the biochemical nature of the common *nod* and *hsn* gene products.

1.6 ROLE OF *RHIZOBIUM* EXOPOLYSACCHARIDES IN SYMBIOSIS

Wild-type *Rhizobium* species produce large quantities of exopolysaccharide (EPS), and the colonies they form are mucoid (Muc^+) in appearance. Some reports suggest that *Rhizobium* EPS may have a role in determining the specificity of symbiosis. Most evidence supporting this possibility came from "binding" experiments (Dazzo *et. al.*, 1976; Kato *et. al.*, 1980). Electron microscopy studies have shown that attachment of the symbiont *Rhizobium* to the host root-hair occurs in a polar fashion, i.e. an end on attachment of the bacteria to the root hair (Sahlman and Fåhraeus, 1963). The attachment of the bacteria to the root hair is thought to be host specific (Dazzo *et. al.*, 1976; Kato *et. al.*, 1980; Zurkowski, 1980). This host-specific adsorption is thought to be determined by the bacterial surface polysaccharides since the inhibition of the adsorption of a *Rhizobium* strain to root hairs by capsular polysaccharide (CPS) from a homologous strain is more pronounced than CPS from heterologous strain (Kato *et. al.*, 1980).

It has been suggested that the host-specificity is determined by selective interactions of lectins on the root surface of host plant with polysaccharides on the cell surface of compatible *Rhizobium* (Albersheim and Anderson-Prouty, 1975). Noninfective rhizobia, therefore are thought not to interact with the host lectin. It was

first suggested by Hamblin and Kent (1973) that lectin from *Phaseolus* can bind *R. phaseoli* to its roots at sites suitable for infection. Lectin prepared from soybean seeds binds specifically to soybean-nodulation bacteria, *R. japonicum* (Bohlool and Schmidt, 1974) and that lectin from clover binds more specifically to *R. trifolii* (Dazzo and Hubbell, 1975), suggesting that these molecules provide a host-*Rhizobium* specificity. The lectin recognition hypothesis (Dazzo, 1980; Dazzo and Gardiol, 1984) states that plant carbohydrate-binding proteins (lectins) have receptor sites that specifically bind compatible *Rhizobium* symbionts to legume root hairs by the recognition of *Rhizobium* surface polysaccharides. Numerous studies present data indicating that the lectin receptor on the symbiont bacteria is the EPS (Bauer, 1981; Carlson, 1982; Dazzo and Gardiol, 1984). However, the EPS may not be the sole lectin receptor on *Rhizobium* as some other surface polysaccharides such as lipopolysaccharide (LPS), may also play a role as lectin receptors on *Rhizobium* (Carlson, 1982; Dazzo and Gardiol, 1984). Lectin binding is claimed to be a host-specific interaction with compatible *Rhizobium*-legume combinations and occurs after a non-specific attachment. The model has only been effectively applied to the *R. trifolii*/clover (Dazzo and Hubbell, 1975) and *R. japonicum*/soybean (Stacey *et. al.*, 1980) symbiosis.

The experimental data, however, have not conclusively proved that the lectin recognition hypothesis is correct. Many results, conflicting with the lectin hypothesis also have been presented. Several *Rhizobium* species bind to lectins isolated from plants which they do not infect or nodulate (Dazzo and Hubbell, 1976; Law and Strijdom, 1977; Wong, 1980; Seegers and LaRue, 1985). Furthermore,

specific attachment or a greater percentage of homologous rhizobia attaching to host roots has not been observed in several instance (Chen and Phillips, 1976; Pueppke, 1984; Vesper and Bauer, 1985). Moreover, lectin pretreatment of *Bradyrhizobium japonicum* does not enhance its attachment to soybean roots (Pueppke, 1984). If lectin mediates attachment to the root, one might expect that lectin pretreatment of *Bradyrhizobium japonicum* could enhance attachment. These results suggest that the lectin requirement may not be absolute and other alternative mechanism (s) for recognition between host and *Rhizobium* may exist.

Yao and Vincent (1969) showed that marked root hair curling occurred only when seedlings were inoculated with the viable homologous cells. *Rhizobium* EPS preparations have been shown to cause non-specific curling and deformation of the root hairs, but do not cause the marked curling (Hubbell, 1970; Vincent, 1974; Yao and Vincent, 1976). Apparently the specific marked curling of the root hair can occur only when viable rhizobia are present (Yao and Vincent, 1976; Ervin and Hubbell, 1985; Canter Cremers *et. al.*, 1986). Ervin and Hubbell (1985) suggest that two fractions, both containing polysaccharide and one also containing protein, are necessary for nonattenuated root hair curling. This suggests that two or more factors are necessary for marked root hair curling.

Several other reports present evidence suggesting that *Rhizobium* EPS may be an important factor in infection and nodulation. Pretreatment of clover (Abe *et. al.*, 1984; Higashi and Ave, 1980) and soybean (Bauer *et. al.*, 1979) seedlings with a low concentration of *Rhizobium* cell surface polysaccharide prior to inoculation results in increased infectivity and nodulation. Pretreatment of white clover

seedlings with as little as 2.5 µg of the CPS or EPS oligosaccharide fragment prior to inoculation with wild-type *R. trifolii* results in a concomitant 70 to 106% increase in infection threads (Abe *et. al.*, 1984). Higher concentrations are not as effective in promoting infection thread formation (Abe *et. al.*, 1984). This suggests that the CPS oligosaccharide fragment is the naturally occurring polysaccharide involved in the infection process.

Another approach to the analysis of the role of cell surface carbohydrates and infection process has been the isolation of non-mucoid (Muc^-), exopolysaccharide-deficient (Exo^-) mutants of different *Rhizobium* strains (Sanders *et. al.*, 1978; 1981; Law *et. al.*, 1982). In general, mutants that are deficient in EPS synthesis are able to form very few, if any nitrogen-fixing nodules on their test plants (Sanders *et. al.*, 1981). In fact, there appears to be a correlation between EPS production and the extent of root hair curling, infection and nodulation efficiency in various spontaneous mutants of *R. leguminosarum* (Napoli and Albersheim, 1980), although this potential correlation is disputed (Sanders *et. al.*, 1981). Spontaneous mutants with an altered capsule synthesis, colony morphology and ability to bind host lectin have been isolated in *R. japonicum* strain 138 (Law *et. al.*, 1982). Three of these mutants failed to form any detectable capsules and still nodulated and attached to soybean roots. The nodulation capacity of several mutants was proportional to the amount of EPS released into the culture medium during exponential growth. It was concluded from these studies that (a) the presence of a capsule physically surrounding the bacterium is not required for attachment or for infection and nodulation, and (b) CPS/EPS synthesis plays an

important role in the infection and nodulation of soybean by *R. japonicum*.

The problem with many of these early studies on Muc$^-$ mutants was that they were done with *Rhizobium* strains in which the genetic basis of the Muc$^-$ phenotype was unclear. More recently, the role of EPS in symbiosis has been also investigated by the isolation of specific, genetically well defined Muc$^-$ or Exo$^-$ mutants of different *Rhizobium* strains (Chakravorty *et. al.*, 1982; Leigh *et. al.*, 1985). A transposon (Tn*5*) -induced mutant of *R. trifolii* which produces no water-soluble EPS (Muc$^-$) causes infection of clover root hair, but the nodules formed are ineffective in nitrogen fixation (Fix$^-$) and less in number (Chakravorty *et. al.*, 1982). Microscopic studies have also shown that while some bacteroid material is formed, this is readily broken down and the bacteria are usually not released from their infection threads (Chakravorty *et. al.*, 1982). A number of transposon (Tn*5*) -induced Exo$^-$ mutants of *R. meliloti* induce non-nitrogen-fixing nodules on *Medicago sativa*. There are no obvious infection thread and intracellular bacteroids in these Fix$^-$ nodules (Leigh *et. al.*, 1985). To some extent, these responses are similar to a "rejection" response of the plant. It appears that the plant reacts to the unencapsulated rhizobia and prevents further spreading of infection. The hybridization and complementation data have shown that these mutations are induced by a single transposon (Tn*5*) insertion which is responsible for both of Exo$^-$ and Fix$^-$ phenotype, suggesting that in *R. trifolii* and *R. meliloti* EPS is not required for nodule initiation but it is essential for the development of a functional nodule (Chakravorty *et. al.*, 1982; Leigh *et. al.*, 1985). More recently, Borthakur *et. al.* (1986) found that a Tn*5*-induced Exo$^-$

mutant of *R. phaseoli* was unaffected in its symbiotic capacity on *Phaseolus* beans but the same mutation prevented nodulation of peas if the resident Sym plasmid was replaced in this Exo$^-$ mutant by a Sym plasmid from *R. leguminosarum*. This appears that the host specific nodulation of peas by *R. phaseoli* requires not only the *hsn* genes of *R. leguminosarum* but also the genes involved in EPS synthesis of *R. phaseoli*.

1.7 AIMS OF THIS STUDY

As indicated in section 1.6, interaction of surface polysaccharides, especially exopolysaccharide, of *Rhizobium* spp. with the host cell surface undoubtedly plays a role in the establishment of the symbiotic state. However, their biological functions and how they regulate their biological activity remain unclear. To gain a clear understanding of the role of *Rhizobium* EPS in symbiosis, it is important to use specific, genetically well-defined exopolysaccharide-synthesis mutants. Early in this study, however, such mutants (transposon Tn*5*-induced Muc$^-$ mutants) had been only isolated from *R. trifolii* (Chakravorty *et. al.*, 1982).

Rhizobium sp. strain NGR234 is unusual in that it is a fast-growing strain able to nodulate a very broad range of plants, including the non-legume plant, *Parasponia andersonii* (Trinick, 1980b; Trinick and Galbraith, 1980). Strain NGR234 shares physiological characteristics with fast-growing *Rhizobium* strain from the tropical tree species *Leucaena leucocephala* and has a symbiotic host range which overlaps slow-growing *Rhizobium* species from the cowpea group of plants. The known host-range of strain NGR234 is

shown in Table 1.2. The broad host-range of strain NGR234 has the advantage of providing a wide range of test plants for assaying mutants for their symbiotic characteristics and therefore excellent opportunities for discriminating between mutations (Morrison, 1984; Rolfe *et. al.*, 1983). Furthermore, strain NGR234, like other wild-type *Rhizobium* strains, produces large amounts of exopolysaccharides. However, the biological function of the EPS produced by this strain in symbiotic associations with host plants had not been investigated. The aims of this study were therefore:

1) To isolate various specific exopolysaccharide-synthesis mutants in strain NGR234, using transposon Tn*5* mutagenesis and to see if these changes on the bacterial cell surface could have fundamental or subtle effects on nodule initiation and development on host plants;
2) to look for correlations between the structure and amount of surface polysaccharides and the ability to form a nitrogen-fixing nodule;
3) to see if the mutants with altered EPS synthesis could induce a hypersensitivity-like plant response by examination of the infection process of the mutants using light and electron microscopy;
4) to investigate the genetic organization and regulation of specific polysaccharide synthesis genes by the establishment of a complementation system to determine whether or not different mutations are in the same gene;
5) to examine the effect of particular cloned polysaccharide genes on regulation of EPS synthesis of homologous or heterologous strains, as well as, their symbiotic properties.

Table 1.1 Cross inoculation groups used to classify the *Rhizobium*-legume symbiosis (after Fred *et. al.*, 1932)

Rhizobium species	Growth rate	pH after culture (a)	Host plants	Plant group
R. melliloti	fast	acid	*Medicago, Melilotus, Trigonella*	Alfalfa
R. Trifolii	fast	acid	*Trifolium*	Clover
R. leguminosarum	fast	acid	*Pisum, Vicia, Lathyrus, Lens*	Pea
R. phaseoli	fast	acid	*Phaseolus*	Bean
R. loti	fast and slow	acid and alkaline	*Lotus, Anthyllis*	Treloil
R. lupini	slow	alkaline	*Lupinus, Ornithopus*	Lupin
R. japonicum	slow	alkaline	*Glycine*	Soybean
Cowpea *Rhizobium*	slow	alkaline	many genera including: *Vigna, Cassia, Arachis, Desmodium, Acacia, Dolichos*	Tropical beans

(a) based on the data of Norris (1965)

Table 1.2 Results of known host-range tests on strain NGR234

Tribe	Sub-tribe	Genus species	Response[a] Nod	Fix	Source[b]
1. Sub-family:	Papilionoideae				
Phaseoleae	Phaseolinae	*Lablab purpureus*	+	+	1, 5
		Macroptilium atropurpureum	+	+	1, 2, 5
		Macroptilium lathyroides	+	+	1, 2,
		Psophocarpus tetragonolobus	+	+	3
		Vigna unguiculata	+	+	1, 2
		Macrotyloma axillaris	-		2
		Phaseolus vulgaris	-		1, 2, 3
	Diocleinae	*Calpogonium caeruleum*	+	+	1
	Clitoriinae	*Centrosema pubescens*	+	+	1
	Cajaninae	*Flemingia congesta*	+	+	1
	Glycininae	*Glycine whightii*	+	+	1, 2
		Glycine ussuriensis	+	+	1, 2
		Glycine tabacina	+	-	2
		Glycine tomentella	-		2
		Glycine Max cv. Bragg	+	-	2
		Glycine max	+	+	1
		Teramnus uncinatus	+	e	2

Table 1.2 Continued

Desmodieae	*Desmodium intortum*	+	+	2, 3, 5
	Desmodium uncinatum	+	e	2, 5
Tephrosieae	*Trphrosia candida*	+	+	1
Aeschenomenae	*Stylosanthes hamata*	+	+	3
	Stylosanthes humilis	-		2
	Arachis hypogea	+	-	2, 3
Robineae	*Sesbania grandiflora*	-		1
Bossiaea	*Crotolaria pubescens*	-		1
Genisteae	*Lupinus augustifolins*	-		1
Vicieae	*Pisum sativa*	-		1, 2
Trifolieae	*Trilolium repens*	-		1, 2
	Trifolium subterraneum	-		2
	Trifolium dubium	-		2
	Medicago sativa	+		1
		-		2
2. Sub-family Mimosoideae				
Mimoseae	*Mimosa invisa*	-		1
	Leucaena leucocephala	+	+	1, 2, 5
Acacieae	*Acacia farnesiana*	+	-	1

Table 1.2 Continued

3. Sub-family Caesalpinioideae				
Cassieae	*Cassia tora*	-		1
4. Non-legumes				
Family Ulmaceae	*Parasponia andersonii*	+	-	2, 4, 5

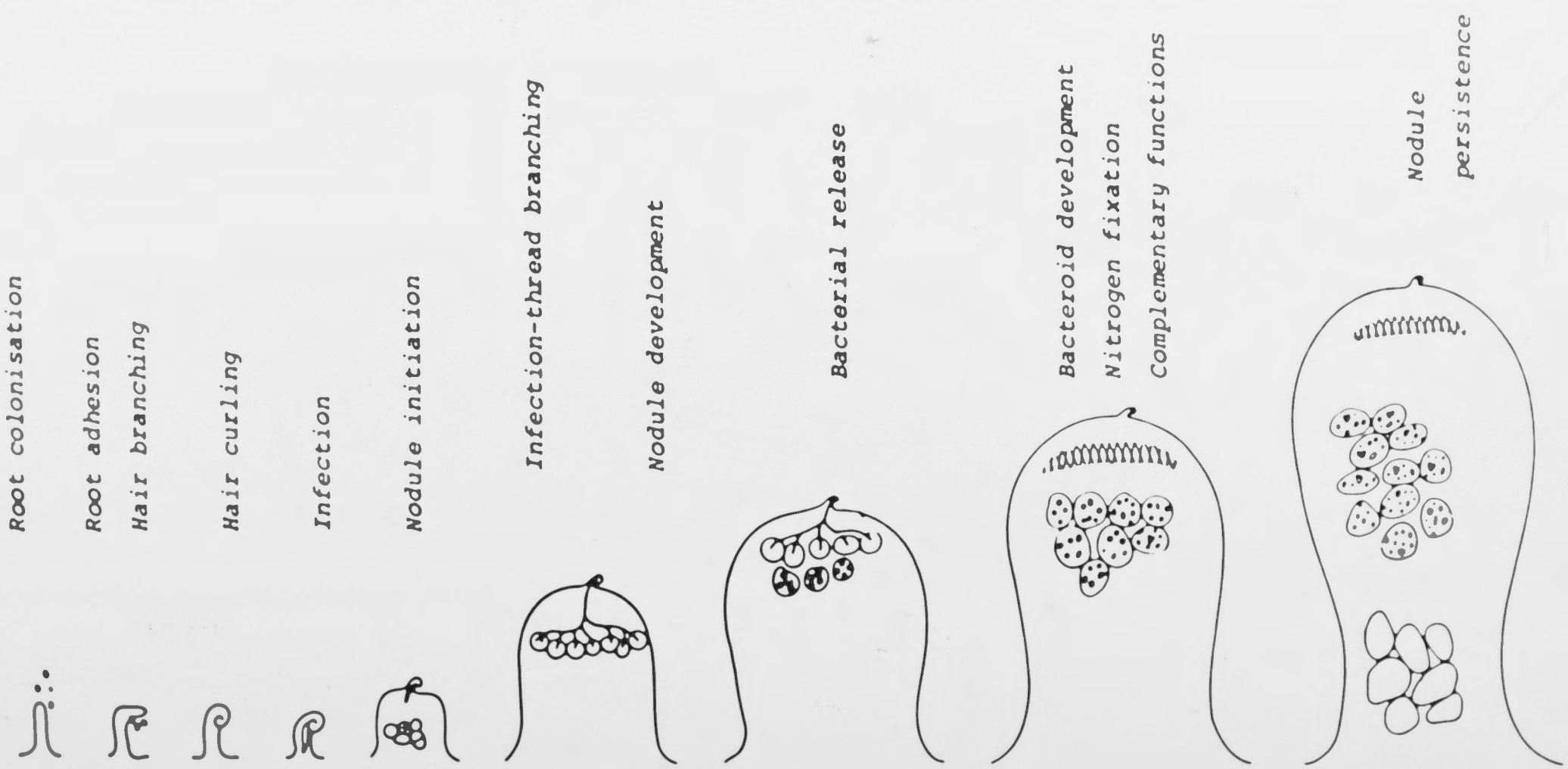
Note

a. Nod^+ means nodulation, Nod^- means no nodulation. Fix refers to nitrogen fixation; + is fully effective, e is partially effective, - means no nitrogen fixation.

b. Sources: 1. Trinick (1980); 2. Morrison (1984); 3. W. Broughton, personal communication (listed in Morrison (1984)); 4. Trinick and Galbraith (1980); 5. Personal observations.

Figure 1.1. A phenotypic key devised by Vincent (1980) and Rolfe *et al.* (1980a) to describe the stages of the infection process.

Roc	Roa	Hac	Inf	Inf	Inb	Nod	Nod	Bad	Nif
	Hab			Noi			Bar	Nif	Cof
	Hac							Cof	Nop



CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 *Bacterial strains and plasmids*

Bacterial strains are listed in Table 2.1. Plasmids used are listed in Table 2.2.

2.1.2 *Plant species and varieties*

These are listed in Table 2.4.

2.2 METHODS

2.2.1 *Plant culture*

Depending upon the particular plant the method of cultivation varied. The methods used were as follows:

a. Plate culture (Rolfe *et. al.*, 1980a): This method was used for assays on siratro, *Desmodium intortum, and D. uncinatum*. *Desmodium* seeds were washed firstly with water, then with 80% ethanol for 3-5 min in a sterile petri dish. They were then washed a further three times with water, followed by a 15 minutes treatment with 5%

hypochlorite. The seeds were kept immersed and frequently stirred to ensure adequate exposure to the sterilant. Hypochlorite was decanted and seeds washed five times with sterile water. Seeds were placed on BMM (see 2.2.7 e) plates which were wrapped in aluminium foil and incubated vertically at 28°C for one to two days. Germination on a growth medium such as BMM allowed the detection of contaminated seedlings which were discarded prior to nodulation tests. Siratro seeds were treated in concentrated sulphuric acid for 5 minutes to scarify the seed coat. They were washed in tap water and then surface sterilised by treatment for 15 minutes in 6.25% hypochloride. Seeds were washed five times with distilled water and placed on BMM plates before being covered with soft agar. After the soft agar has set, the plates were wrapped in aluminium foil and incubated horizontally at 29°C for one day. *Parasponia* seed germination (Bender and Rolfe, 1985) was done as follows: Seeds were removed from fruit by gentle rubbing between wire gauze and a rubber stopper, seeds were then shaken in 96% ethanol for 10 minutes to remove traces of the oily fruit. Seeds were washed five times with distilled water then shaken in 12.5% sodium hypochlorite for 30 minutes. Seeds were washed five times with sterile distilled water. Both ethanol and sodium hypochlorite treatments were carried out in a 250-ml conical flask shaken at 200 rpm on a rotary shaker. Seeds were washed using a sintered glass filter and vacuum flask. Seeds were then arranged on the surface of a distilled water agar plate and secured with a single drop of soft agar pre-cooled to 55°C. The plate was sealed with Nescofilm (Nippon Shoji Kaishi Ltd., Osaka, Japan) which was pierced with a small hole for gas exchange. The plate was placed vertically in a growth cabinet with Grolux WS fluorescent tubes as a light source

temperature variation of 25°C (night) to 35°C (day). After eight days incubation, seed germination was about 91%.

Plates containing F or P (specific for *Parasponia*) agar medium (30 ml per plate) were prepared for inoculation by streaking a loopful of *Rhizobium* cells over about half the surface of a plate. Two (siratro) or three (*Desmodium, Parasponia* seedlings (germinated as above) were placed on the plate just above the line of the streaked bacteria. To facilitate adhesion of the plants to the agar, plates were left horizontally for about an hour before being sealed with Nescofilm strips. Two or three small slits were made in the Nescofilm at the top of the plate to allow for gas exchange. Plates were incubated vertically and stacked in rows in a growth cabinet with a 26°C, 18-h day and 20°C, 6-h night for siratro and *Desmodium* plants and with a constant temperature of 28°C for *Parasponia* (light intensity averaged about 300-350 $\mu E\ m^{-2}\ s^{-1}$).

b. Plastic cylinders: This method was used for *Leucaena* plant assays. *Leucaena* seed germination was the same as siratro except that the seeds were treated in concentrated sulphuric acid for 10 minutes. *Leucaena* nodulation assays were done with sterile 110 mm plastic cylinders which contained 75 ml of F agar medium made up as a slope. A seedling was placed on top of the agar slope, inoculated with bacteria and a sterilized transparent plastic bag used to cover the container. The plastic bags were removed after the inoculated plants has grown for 4 days. The open end of the plastic cylinder was sealed with Nescofilm except for the protruding stem and leaves. Plant growth conditions were the same as siratro.

c. *Lablab* test: *Lablab* seed germination was the same as *Desmodium* except that the BMM plates which contained seeds were incubated horizontally. Germinated seeds were transferred to 250 ml conical flasks containing 200 ml of solid F medium and incubated at room temperature in the dark for 2 days. Each plant was inoculated with a 10 ml aliquot of bacterial suspension (10^6 ml^{-1}). Plants were cultivated under the same conditions as siratro.

d. Plant media:

Gibson's Trace Elements Solution (Gibson, 1963)

H_3BO_3	2.86	g l^{-1}
$MnSO_4 \cdot 4H_2O$	2.03	g l^{-1}
$ZnSO_4 \cdot 7H_2O$	220	g l^{-1}
$CuSO:_4 \cdot 5H_2O$	80	mg l^{-1}
$H_2MoO_4 \cdot 1H_2O$	90	mg l^{-1}

Fåhreaus medium (F) (modified) (Vincent, 1970)

$CaCl_2 \cdot 2H_2O$	100	mg l^{-1}
$MgSO_4 \cdot 7H_2O$	120	mg l^{-1}
KH_2PO_4	100	mg l^{-1}
$Na_2HPO_4 \cdot 12H_2O$	150	mg l^{-1}
Ferric citrate	1.5	mg l^{-1}
Gibson's trace elements	1	ml l^{-1}
pH	7.0	

Plant nutrient medium (P) (Bender and Rolfe, 1985)

K_2HPO_4	200	mg l^{-1}
$MgSO_4 \cdot 7H_2O$	200	mg l^{-1}
$CaSO_4 \cdot 2H_2O$	200	mg l^{-1}

FeNaEDTA	40	mg l^{-1}
KCl	40	mg l^{-1}
$CuSO_4 \cdot 5H_2O$	0.1	mg l^{-1}
$ZnSO_4 \cdot 7H_2O$	0.3	mg l^{-1}
$MnSO_4 \cdot H_2O$	1.7	mg l^{-1}
H_3BO_3	1	mg l^{-1}
$Na_2MoO_4 \cdot 2H_2O$	0.1	mg l^{-1}
$CoCl_2 \cdot 6H_2O$	0.02	mg l^{-1}
KNO_3	40	mg l^{-1}
pH	7.0	

Soft agar

1 M $MgSO_4$	10	ml l^{-1}
agar	6	g l^{-1}

The media were autoclaved at 15psi for twenty minutes at 120°C.

2.2.2 *Acetylene reduction*

Whole plant assays were done on all plants except *lablab*. The plant was removed from the culture media and placed in a 28 ml glass scintillation vial with 0.3 ml of sterile water and a piece of filter paper. The wet filter paper maintained humidity. The vials were capped with Suba seals which had been washed and dried overnight at 60°C to remove any residual ethylene. The gas phase was augmented by the addition of 2 ml of pure acetylene and the vials were incubated at 26°C under illumination (300 µE m^{-2} s^{-1}) in a growth cabinet. Samples of 200 µl were taken at hourly intervals and assayed for ethylene by

gas chromatography, with a Poropak R-80-100 column at 45°C with nitrogen gas as the mobile phase and a hydrogen flame ionization detection system. Quantitation of ethylene production was done by reference to calibrated standards. Rates were expressed as n mole acetylene reduced per hour per gram fresh nodule weight or per plant.

Lablab plants had their root systems excised and placed in 200 ml air tight jars capped with Suba seals. Acetylene (20 ml) was injected and the production of ethylene monitored as above.

2.2.3 *Preparation of exopolysaccharides*

a. Growth in liquid medium: Strains were grown to different stages of growth in M liquid medium (see 2.2.7 e). The cultures of the mutant and parent strain were harvested at mid and late exponential growth phase and in stationary growth phase. The cultures were centrifuged at 15,000rpm for 30 minutes and the supernatant was tested for exopolysaccharides (EPS) by measuring hexose and uronic acids.

b. Growth on solid medium: Strains were grown to different stages of growth on BMM selective medium. The bacteria were scraped off one to two selective medium plates and were suspended in 50 ml of sterile 10 mM phosphate-buffered saline. The suspensions were shaken at 300 rpm for 1 hr and then centrifuged at 15,000 rpm for 30 minutes. The supernatants were tested for EPS by measuring hexose.

2.2.4 *Measurement of EPS*

a. Hexose: A modified anthrone method (Shields and Burnett, 1960) was used for the determination of hexose. Anthrone solution (0.2% anthrone W/V in 96% sulfuric acid) was made up at least 4 hours before use and prepared fresh each day. 8 ml of anthrone solution was added dropwise to 4 ml of the EPS-containing solution, previously cooled to under 4°C by immersion in an ice bath for 45 minutes. After mixing the contents, the firmly stoppered tubes were immersed in a hot water bath at 92°C for 8 minutes. On withdrawal, the tubes were re-immersed in the ice bath to stop the reaction. The solutions were transferred to cuvettes and after 30 minutes their absorbancies read at 585 μm in an LKB Biochrom Ultrospec 4050. All estimations were done in duplicate. Glucose was used as the standard.

b. Uronic acid (Bitter and Muir, 1962): 4 ml of sulfuric acid reagent (0.025 M sodium tetraborate.$10H_2O$ in sulfuric acid) was placed in tubes fixed in a rack and cooled to 4°C; 1 ml of the sample or standard was carefully layered on to the acid. The tubes were closed with Teflon stoppers and the rack shaken at first gently, then vigorously with constant cooling. The tubes were then heated for 10 minutes in a vigorously boiling water bath and cooled to room temperature. Carbazole reagent (0.125% carbazole in absolute ethanol, 0.2 ml) was then added; the tubes were shaken again, heated in the boiling bath for a further 15 minutes, and cooled to room temperature. The optical density (OD) was then read at 530 μm in a LKB Biochrom Ultrospec 4050. D-glucuronic acid was used as the standard.

2.2.5 *Treatment of plant roots with EPS or oligosaccharide*

The purified EPS and its derived oligosaccharide from strain NGR234 were kindly supplied by Drs M. Batley and J. Redmond. Sterile *Leucaena* seedlings germinated on BMM agar were transferred to the slope of the agar in a plastic cylinder (2.2.1 b) and allowed to settle to the agar surface for 10 hours. Aliquots (5 µl) of sterile stock solutions (5mg/ml) of EPS or its derived oligosaccharide were dropped along the length of 3 day-old seedlings. This concentration was high enough to restore the abilities of Exo^- mutants of NGR234 to effectively nodulate *Leucaena leucocephala* and siratro (see Chapter 7). These acidic saccharides were added either simultaneously or 24 hours or 48 hours before or after inoculation with rhizobia.

2.2.6 *Measurement of protein content of bacterial pellets*

Measurement of protein content of bacterial pellets was according to the method described by Lowry *et. al.* (1951).

a. Reagents: Reagent A, 2% Na_2CO_3. Reagent B, 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate. Reagent C, alkaline copper solution. Mix 50 ml of Reagent A with 1 ml of Reagent B. Discard after 1 day. Reagent D, diluted Folin reagent (50% Folin reagent in distilled water).

b. Procedure: Bacterial pellets were suspended in 0.1 ml of 1 M NaOH and the suspensions were transferred into glass tubes. The tubes were closed with Teflon stoppers and boiled for 20 minutes. After boiling, 0.4 ml of H_2O and 1 ml of Reagent C were added, mixed well

and allowed to stand for 10 minutes or longer at room temperature. Then 0.1 ml of Reagent D was added very rapidly and mixed within a second or two. After 30 minutes, the sample was read at 500 µm in a LKB Biochrom Ultrospec 4050. Bovine serum albumin was used as standard.

2.2.7 *Bacterial matings*

a. Filter matings: Recipient and donor strains were grown to mid-log phase on appropriate media, either solid or liquid and resuspended in 4 ml of sterile water. *Rhizobium* recipients were always grown on TY medium, whilst *E.coli* strains were grown on LB medium. Bacterial suspensions (2 ml) were mixed in a test tube by vortexing and filtered through a sterile 0.45 µm nitrocellulose filter (Millipore Inc., Detroit, U.S.A.). The bacteria remained on the filter which was placed on solid medium. For *Rhizobium* the mating was done on TY medium for 6 hours at 29^{o}C whilst for *E.coli* it was done at 37^{o}C. The filter was removed from the agar surface after this time and the cells washed off by vortexing in 4 ml of sterile water in a sterile screw-capped bottle. The cell suspension was diluted serially ten-fold and 0.1 ml aliquot plated onto appropriate media to select for transconjugants or to ascertain the viable count of recipient and donor cells. Transfer frequencies were presented as the ratio of transconjugants to total recipient cells. Donor cells were usually at about the same number as recipient cells.

b. Patch matings: This semi-quantitative method was used when transfer frequencies were expected to be high. Donor and recipient

cells, grown as described above, were resuspended in 0.5 ml of sterile water. *E.coli* donors were normally grown on TY medium for crosses with *Rhizobium* to avoid potential toxicity of the salt in LB medium towards certain rhizobia, notably strain NGR234 derivatives. Donor and recipient cell suspensions (0.05 ml) were mixed in a test tube by vortexing and the mixture dried onto one half of the surface of a TY plate. Control spots of pure donor and recipient suspensions were dried on the other half of the TY plate. The cultures were incubated overnight at 29^{o}C before being replica plated onto a series of different media to select for transconjugants, donor alone and recipient alone. The rate of transfer could be estimated by the number of transconjugants which grew. High transfer frequencies gave confluent growth on selective media. Transconjugants from this type of mating have to be rigorously purified on selective media since the chances of donor contamination are higher.

c. Spot matings: This type of mating was used to test for the re-transfer of plasmids from a large number of donors to a single recipient. A loopful of donor cells was mixed with a loopful of recipient cells on the surface of a TY plate. Up to 20 mixed spots could be located on a plate. The "mating plates" were incubated at 29^{o}C overnight before being replica plated onto selective media. *E.coli* transconjugants were selected at 37^{o}C and *Rhizobium* transconjugants were selected at 29^{o}C. Control spots of plasmids of known transmissibility were always included. Strains containing transferrable plasmids could easily be identified since recipient transconjugants only grew on the selective media at positions corresponding to those strains on the master plate.

d. Transposon mutagenesis with the plasmid pSUP1011: The plasmid pSUP1011 has a gene for chloramphenicol resistance (Cm^r), a site for mobilization (Mob) into Gram-negative bacteria, and carries the transposon Tn*5* (Simon *et. al.*, 1982). The plasmid is derived from pACYC184, a plasmid with a *Col*E1-like DNA replication system (Chang and Cohen, 1978). This plasmid cannot be maintained in *Rhizobium*. The Mob site is the Ori-T region of plasmid RP4. This Mob region can interact with the transfer function provided by RP4, *in trans*, to promote conjugal transfer of whatever molecule contains the Mob site. The *E.coli* strain carrying pSUP1011, SM10, has the Ap^s, Tc^s, Km^r derivative of RP4, containing a Mu prophage, integrated in the *E.coli* chromosome. This integrated plasmid provides the necessary transfer functions to mobilise pSUP1011. After pSUP1011 has entered the *Rhizobium* cell the Tn*5* copy can "escape" by transposition while the donor plasmid is lost.

Donor [SM10(pSUP1011)] and recipient (ANU280) were grown on LB and TY slopes respectively and were mated on filters. The frequency of transfer of Km^r was about 10^{-6}. Matings with the donor strain SM10 gave no transfer of Km^r. This control mating was done to check that the RP4::Mu(Ap^s, Tc^s Km^r) plasmid of SM10 did not transfer to strain ANU280. Plates containing several hundred or several thousand colonies were examined carefully for any colonies with altered morphologies. Colonies which were rough (Muc^- phenotype), semi-rough or translucent were picked and purified on the selective media. Two hundred such colonies were tested for Cm^r, the other marker on pSUP1011. All colonies were chloramphenicol sensitive (Cm^s) suggesting that these mutants were induced by Tn*5* insertions rather than the plasmid insertions.

e. Bacterial media:

Luria Broth (LB) (Miller, 1972)

Bacto-tryptone	10	g	l^{-1}
Yeast extract	5	g	l^{-1}
NaCl	5	g	l^{-1}
pH	7.5		

Gamborg's Trace Elements Solution

(Gamborg and Eveleigh, 1968)

$Mnso_4 \cdot 4H_2O$	10	g	l^{-1}
H_3BO_3	3	g	l^{-1}
$ZnSO_4 \cdot 7H_2O$	3	g	l^{-1}
$Na_2MoO_4 \cdot 1H_2O$	250	mg	l^{-1}
$CuSO_4 \cdot 5H_2O$	250	mg	l^{-1}
$CoCl_2 \cdot 6H_2O$	250	mg	l^{-1}

Bergersen's Modified Medium (BMM) (Bergersen, 1961)

$Na_2HPO_4 \cdot 12H_2O$	360	mg	l^{-1}
$MgSO_4 \cdot 7H_2O$	80	mg	l^{-1}
$FeCl_3$	3	mg	l^{-1}
$CaCl_2$	40	mg	l^{-1}
Mannitol	3	mg	l^{-1}
Thiamine	2	mg	l^{-1}
Biotin	0.2	mg	l^{-1}
Sodium glutamate	500	mg	l^{-1}
Yeast extract	500	mg	l^{-1}
Gamborg's trace elements	1	ml	l^{-1}
pH	7.0		

BMM plus Mannitol (BMM+Man) (Gresshoff and Rolfe, 1978)

As for BMM but with the addition of 36 g l^{-1} mannitol.

Tryptone Yeast (TY) (Beringer, 1974)

Bacto-tryptone	5 g l^{-1}
Yeast extract	3 g l^{-1}
$CaCl_2 \cdot 2H_2O$	0.9 g l^{-1}
pH	7.0

Trifolii Medium Yeast (TMY) (Skotnicki and Rolfe, 1979)

$(NH_4)_2SO_4$	250 mg l^{-1}
KH_2PO_4	150 mg l^{-1}
$MgSO_4$	200 mg l^{-1}
NaCl	100 mg l^{-1}
$NaMoO_4$	25 mg l^{-1}
$CaCl_2 \cdot 2H_2O$	250 mg l^{-1}
$FeSO_4 \cdot 7H_2O$	2.5 mg l^{-1}
Sucrose	5 g l^{-1}
Arabinose	5 g l^{-1}
Thiamine	1 mg l^{-1}
Biotin	0.5 mg l^{-1}
Nicotinic acid	1 mg l^{-1}
Pyridoxine-HCl	1 mg l^{-1}
Gamborg's trace elements	1 ml l^{-1}
Yeast extract	1 g l^{-1}
pH	7.1

Yeast Extract Medium (Y)

K_2HPO_4	500	mg	l^{-1}
KH_2PO_4	500	mg	l^{-1}
$MgSO_4$	200	mg	l^{-1}
$CaCl_2 \cdot 2H_2O$	40	mg	l^{-1}
Yeast extract	1	g	l^{-1}
pH	6.8		

Minimal Medium (M)

K_2HPO_4	1	g	l^{-1}
KH_2PO_2	1	g	l^{-1}
$FeCl_3 \cdot 6H_2O$	10	mg	l^{-1}
$MgSO_4 \cdot 7H_2O$	250	mg	l^{-1}
$CaCl_2 \cdot 6H_2O$	100	mg	l^{-1}
Sodium glutamate	1.1	g	l^{-1}
Mannitol	10	g	l^{-1}
Thiamine	1	mg	l^{-1}
Biotin	0.5	mg	l^{-1}
Nicotinic acid	1	mg	l^{-1}
Pyridoxine . HCL	1	mg	l^{-1}
Gamborg's trace elements	1	ml	l^{-1}
pH	7.2		

The media were autoclaved at 15psi for twenty minutes at 120°C.

f. Antibiotics: For *Rhizobium* strains the following concentrations of antibiotics were used:

Carbenicillin (Cb)	75	µ	ml^{-1}
Chloramphenicol (Cm)	15	µ	ml^{-1}
Kanamycin (Km)	200	µg	ml^{-1}

Streptomycin (Sm)	400	µg ml^{-1}
Spectinomycin (Sp)	200	µg ml^{-1}
Rifampicin (Rif)	50	µg ml^{-1}
Tetracycline (Tc)	4	µg ml^{-1}

For *E.coli* strains the following concentrations of antibiotics were used:

Km	50	µg ml^{-1}
Rif	50	µg ml^{-1}
Tc	15	µg ml^{-1}
Ampicillin	100	µg ml^{-1}

These antibiotic solutions were filtered through a sterile 0.45µm nitrocellulose filter into sterile screw-capped bottles.

2.2.8 *Isolation of bacteria from nodules*

The method of Gresshoff *et. al.* (1977) was used. The nodule was excised from the plant and washed in sterile water several times before being surface sterilised in 1% sodium hypochloride for 10 to 15 minutes. Nodules were then rinsed free of sodium hypochloride and transferred to 0.1 ml of protoplast dilution buffer (PDB). Nodules were crushed with a sterile glass rod and the suspension plated out on BMM+Man solid medium.

PDB consisted of;

Sorbitol	250	mM
Mannitol	250	mM
Di-potassium hydrogen orthophosphate	2	mM
Calcium chloride	2	mM
pH	5.8	

2.2.9 *Preparation of heat-killed ANU265 cells*

Strain ANU265 was grown on BMM agar plates contained 100 µg/ml of spectinomycin for 2 days. The bacteria were scraped off a plate and transferred into a sterile test tube which was then heated in a boiling water bath for 2 hours. To test for survivors, 0.5 ml of heated cells was suspended in 4.5 ml of sterile water and 0.1 ml of suspension was spread on each of five BMM plates. These plates were incubated at 29°C for 6 days and no colonies appeared.

2.2.10 *DNA isolation*

a. Genomic DNA: *Rhizobium* strains were grown in 10 ml of TY medium at 30°C for about 30 hours with shaking. Cells were pelleted by centrifugation and resuspended in 3 ml of 25% sucrose in TE and protoplasted by the addition of 100 µl of 20 mg ml^{-1} lysozyme in water. After 5 minutes at room temperature the cells were lysed by the addition of 3 ml of 0.1% SDS. Proteinase K, 10 mg, was added and the lysate incubated at 65°C for 30 minutes. The lysate was phenol extracted four times with centrifugation to separate the aqueous and organic phases. The lysate was then extracted four times with phenol-chloroform, followed by four extractions with chloroform alone. DNA was ethanol precipitated, dried *in vacuo* and reconstituted in 1 ml of TE buffer.

b. Plasmid DNA: *E.coli* strains carrying plasmids were grown in 50 ml LB medium at 37°C with shaking. A 10 ml aliquot of this culture was used to inoculate 1 litre of LB media and incubated overnight at

37°C with shaking. The cells were harvested by centrifugation at 5,000rpm for 5 minutes at 0-4°C. The cell pellet was resuspended in 10 ml 25% sucrose in TE and transferred to 40 ml polypropylene tubes. Freshly prepared lysozyme (30 mg in 1 ml of water) was added, followed by 5 ml 0.5 M EDTA pH 8.0. After 5 minutes the cells were lysed by the addition of 15 ml of 1% Triton-X100. The lysate was centrifuged at 200,000 rpm for one hour at 4°C. The supernatant was decanted and made 3% with respect to sodium chloride and 12.5% with respect to polyethylene glycol 6000(PEG) and maintained on ice for two hours. The DNA was pelleted by centrifugation at 5,000 rpm for 5 minutes. The pellet was then dissolved in 5 ml TES buffer and 8 g of caesium chloride was added. The caesium chloride was dissolved by gentle rocking of the tube and the solution was centrifuged at 18,000 rpm for 30 minutes. A pellicle of PEG was removed from the tube with a spatula. A 1.5 ml aliquot of TES and 0.6 ml ethidium bromide (10 mg ml^{-1}) was added to obtain a final density of 1.6 g ml^{-1}. The solution was centrifuged in self-sealing Beckman polypropylene tubes at 40,000 rpm for 40 hours at 18°C in a Beckman 50Ti rotor. DNA was visualised with a UV lamp and plasmid DNA was extracted through a side-puncture of the centrifuge tube. Ethidium bromide was removed from the DNA by extraction against butanol. The DNA was dialysed against three changes of TE buffer and then ethanol precipitated and reconstituted to a concentration of 1 mg ml^{-1}.

c. Boiling method (Holmes and Quigley, 1981): Single colonies were picked and grown overnight in 2 ml LB at 37°C (for *E.coli*) and in 2 ml of TY at 30°C (for *Rhizobium*) with shaking. A 1 ml aliquot of cells was centrifuged in an Eppendorf tube (Eppendorf centrifuge) for

30 secs. The pellet was resuspended in 200 µl of STET buffer (8 mg ml^{-1} glucose, 0.1 mg ml^{-1} Triton X100, 50 mM Tris-HCl, 50 mM EDTA, pH8.0) and the solution boiled for 40 secs. The mixture was centrifuged for 15 minutes to pellet the cell debris and the supernatant removed to fresh tubes. DNA was precipitated twice, and redissolved in 20 µl of TE.

d. Alkaline lysis (Birnboim and Doly, 1979): A 1.5 ml sample of an overnight culture of *E.coli* grown in LB medium was pelleted and resuspended in 100µl of a cold solution of 50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA with lysozyme at 6 mg ml^{-1}. After the cells were protoplasted they were lysed by the addition of 180 µl of cold 0.2 N NaOH, 1% SDS solution. After complete lysis 200 µl of 3 M potassium, 5 M acetate pH 4.8, was added. This solution made a flocculent white precipitate between chromosomal debris bound with SDS and potassium. The precipitate was spun down, the supernatant removed and phenol-chloroform extracted. Ethanol, 1 ml, was added and after about 5 minutes at room temperature the DNA was collected by centrifugation. The pellet was washed in 70% ethanol, then dried in absolute ethanol. After removing the ethanol the DNA was redissolved in 50 µl of water. For a multicopy plasmid this procedure yielded about 2 µg DNA.

e. Phenol-chloroform extraction of DNA: Organic solvent extraction was used to remove protein from DNA. The crude DNA preparation was mixed gently by inversion with an equal volume of "phenol" (phenol saturated with TE buffer) and the phases separated by centrifugation. The aqueous phase was extracted again, eventually

resulting in a perfectly clear aqueous phase after repeated extractions. The DNA preparation was then extracted with "phenol-chloroform" (an equal volume of phenol and chloroform) to remove remaining protein. The DNA was then extracted with pure chloroform to remove residual phenol. DNA was then ethanol precipitated by adding 0.1 volume of 3 M sodium acetate pH 6 and 2.5 volumes of ethanol. After mixing, large molecular weight DNA would precipitate at room temperature. Dilute preparations of DNA or small molecular weight DNA pellet was washed in 70% ethanol. Ethanol was removed *in vacuo*. Dry DNA was redissolved in TE buffer to a concentration of 1 mg ml^{-1}, assessed by measuring ultraviolet light absorbance at 260 nanometres. The DNA was stored at 4°C.

f. Buffer used for DNA isolation and storage:

TE buffer consisted of;

Tris-HCl	10	mM
EDTA	1	mM
pH	8.0	

TES buffer was TE with 100 mM sodium chloride.

The buffers were autoclaved at 15psi for 20 minutes at 120°C and then used for DNA isolation.

2.2.11 *Electrophoresis of nucleic acids*

a. Modified Eckhardt gels: A modified Eckhardt method (Plazinski *et. al.*, 1985) was used for large plasmid visualization. A horizontal 0.7% agarose gel was made using a double comb system. When

the gel was set the comb nearest the cathode was removed and the wells were filled completely with a solution of 0.4% agarose containing 1% SDS. After 1 hour the second comb (nearest the anode) was removed and 40-80 µl of cell sample was loaded into the vacant wells. Samples were prepared as follows; overnight cultures were diluted (1:10 to 1:30) in liquid medium and grown for another 2-3 hours with shaking at 30°C (for *Rhizobium*) or 37°C (for *E.coli*). Samples of 300 to 800 µl of each bacterial suspension were spun down and washed with 400 µl of 0.1% sarkosyl in TE buffer. Each pellet was gently suspended in 40 to 80 µl of 1 mg ml^{-1} lysozyme, 100 µg ml^{-1} RNA'ase A, 160 mg ml^{-1} Ficoll-400,000 in TBE buffer and left for 10 minutes at room temperature. Before loading, samples were gently suspended by pipetting and a 8 µl of Proteinase K (10 mg ml^{-1}) solution was added into each well and left for 5 minutes at room temperature. The electrophoresis buffer (TBE) was poured into an electrophoresis tank up to the edge of the gel but not covering the gel. The gel was run at 10 mA for 2 hours at room temperature. Then additional buffer was added to the gel tank so as to completely cover the gel by about 3 mm of buffer solution. The electrophoresis system was then run at room temperature at 45 mA for 8-10 hours.

b. Agarose gels of restricted DNA: Horizontal slab gels of 140 x 160 x 4 mm dimensions made with 0.8% agarose (SeaKem, Marine Colloids, Inc.) in TBE buffer were routinely used for analysis of digested DNA. Samples were run with the addition of dye and heated at 65°C for 5 minutes prior to loading. Gels were run for 10 to 15 hours at 45 mA, 30V. All gel types were stained by soaking in 4 µg ml^{-1} ethidium bromide for 20 minutes followed by destaining in water for 20

minutes. DNA was visualised by exposure to ultraviolet light, 250 nanometres wavelength, using a transilluminator and photographed on Kodak type 107 or 667 film using a Kodak Wratten-gelatin filter No. 28. Molecular weight markers were bacteriophage lambda *c*I857 S*am*7 DNA cut with various enzymes.

c. Electrophoresis buffers: Tris-Borate-EDTA (TBE) buffer (Eckhardt, 1978) was routinely used for agarose gel electrophoresis of DNA. The buffer was prepared as concentrate and diluted twenty times prior to use.

TBE

Tris-acetate	89	mM
Boric acid	8.9	mM
Ethylenediamine tetraacetic acid (EDTA)	1	mM
pH	8.0	

Gel sample buffer

Glycerol	80	mg ml^{-1}
EDTA	100	mM
Bromophenol Blue	0.3	mg ml^{-1}
pH	8.0	

2.2.12 *Southern hybridization procedures*

a. Gel blotting: This method (Southern, 1975) was used to identify homologous DNA resolved in agarose gels (Section 2.2.11 b.). If the DNA was of molecular size greater than 10 to 20 kb the gel was

first soaked in 0.25 N HCL for 20 minutes to cause partial depurination of the DNA. This step was omitted for small molecules. Gels were soaked in 0.5 M NaOH, 0.5 M NaCl for 45 minutes at room temperature to denature the double stranded DNA. The gel was then soaked for 30 minutes in 0.5 M Tris-HCl pH 7.2, 2 M NaCl to neutralise the base and to maintain denaturing conditions. The gel was then placed on filter paper wicks which were in contact with a solution of 20 x SSC. Wetted nitrocellulose was placed on the uppermost surface of the gel and two layers of wetted filter paper were placed on top. A wad of blotting paper was added to start blotting the denatured DNA onto the nitrocellulose. After about 12 hours the blotting paper absorbed about 100 ml of the 20 x SSC. The nitrocellulose was removed, briefly washed in 2 x SSC, dried in the air, then baked *in vacuo* for 2 hours at 80°C. The nitrocellulose "filter" was then ready for hybridization.

b. Buffers for Southern hybridization:

DNA denaturation buffer

Sodium hydroxide	0.5	M
Sodium chloride	0.5	M

Tris-salt buffer

Tris-HCL	0.5	M
Sodium chloride	2.0	M
pH	7.2	

Sandard saline citrate (SSC), 20 X concentrate

Sodium chloride	3.0	M
Tris-sodium citrate	0.3	M

c. Radioactive probes: Plasmid DNA (1 µg) was restricted with the enzyme *Hae*III and denatured by boiling for 2 minutes with 2 µg of random primers (8-12 nucleotide fraction of DNA'ase I treated calf thymus DNA) in 20 µl and cooled on ice for 2 minutes. Deoxynucleotides, dCTP, dGTP, and dTTP (1 µl, 20 mM) and 3 µl of radioactively labelled dATP (3,000 Ci $mMol^{-1}$, 10 mCi ml^{-1}, BRESA) were added to the DNA and the reaction mix made up to 30 µl in *Hae*III buffer. The sample was then incubated with 1 unit of DNA polymerase I (large fragment) at 37°C for 30 minutes and the reaction stopped by phenol extraction. The phenol phase was washed twice with 50 µl of water and the combined fractions passed over a Sephadex G-50 column (bed volume 4 ml, medium grade G-50) with TES running buffer. Radioactively labelled DNA was monitored with a Geiger counter and eluted just after the void volume of the column (1 ml) whilst the unincorporated triphosphates were retarded on the column. The probe volume was usually about 600 µl.

d. Hybridization conditions: Nitrocellulose filters were hybridized with probes in plastic bags. Firstly, the filter was wetted with pre-boiled hybridization solution and pre-hybridized for one hour at 65°C. This procedure tends to reduce non-specific binding of the probe to the filter. After pre-hybridization, the hybridization solution was reduced in volume to about 1 ml per 100 cm^2 of filter. The probe DNA was denatured by boiling for two minutes and then added to the hybridization bag. The bag was sealed and the probe evenly distributed over the surface of the filter. The probe was allowed to hybridize with the filter at 65°C for varying times, usually 16 hours. After hybridization, the filter was washed four

times in 2 x SSC for about 30 minutes each time at room temperature. If too much non-specific binding of the probe had occurred the filter was re-washed at 65°C for one hour in 0.1 x SSC and 1% SDS. Filters were then dried in air prior to being exposed to X-ray film (Kodak XRP-5 and XAR-5 type film) at -80°C with Cronex (DuPont) "Lighting Plaus" intensifying screens for varying times, usually one to two days.

e. *Hybridization buffer*:

Sodium dodecylsulphate (SDS)	0.1	mg ml^{-1}
Herring sperm DNA (Sigma)	0.2	mg ml^{-1}
Polyvinylpyrrolidene	0.2	mg ml^{-1}
Hepes buffer (Sigma)	50	mM
Ficoll-400 (Sigma)	0.2	mg ml^{-1}
20 x SSC	150	ml l^{-1}
BSA	0.2	mg ml^{-1}
pH	7.0	

2.2.13 *Cloning procedures*

a. Ligation: DNA was ligated in the presence of *Hae*III or TA buffer with 0.5 mM ATP and 10^4 Units of T4 DNA ligase. Ligations were done at 16°C overnight.

b. Transformation: Competent cells (*E.coli* strain RR1, prepared by the method of Morrison, 1979) were thawed on ice prior to transformation. Ligated DNA, diluted to a final volume of 100 µl, was added to 200 µl of thawed cells and left on ice for thirty minutes.

The cells were heated in a water bath at 42°C for 2 minutes, and then inoculated into 5 mls of LB media (at 37°C). The solution was incubated at 37°C for one hour without shaking. Cells were concentrated by centrifugation, resuspended in 1 ml LB and plated onto appropriated selective antibiotic media.

c. Molecular cloning of Tn*5*-induced mutant gene: The total DNA isolated from the Exo$^-$ mutant ANU2811 was digested with restriction enzyme *Eco*RI. 2 µg of the *Eco*RI restricted *Rhizobium* DNA was ligated to 1µg of *Eco*RI-cleaved pBR322 DNA and the recombinant plasmids transformed into *E.coli* RR1 as described in section 2.2.13b. The selection of clones carrying the Tn*5* and flanking *Rhizobium* sequences was carried out by plating the transformation mixture on LB plates containing 50µg ml^{-1} of kanamycin. A recombinant plasmid containing a 6.3kb *Eco*RI fragment of ANU2811 was designated as pE2811. Plasmid pE2811 carried 0.6kb *Rhizobium* DNA sequence since the molecular weight of Tn*5* is 5.7kb.

d. Buffers for enzymology:

TA buffer (O'Farrell *et. al.*, 1980) was used for all restriction endonuclease reactions. A ten times concentrate was prepared by mixing 0.8 ml of solution A and 0.1 ml each of solution B and C, where;

Solution A

Tris-acetate	5.0	mg ml^{-1}
Potassium acetate	8.1	mg ml^{-1}
Magnesium acetate	2.5	mg ml^{-1}
pH	7.9	

Solution B

Dithiothreitol (DTT)	50	mM

Solution C

Bovine serum albumin (BSA)	10	mg ml^{-1}

The final concentrations used in restriction reactions were;

Tris-acetate	33	mM
Potassium acetate	66	mM
Magnesium acetate	10	mM
DTT	500	µm
BSA	100	µg ml^{-1}

HaeIII buffer was used for ligation reactions and making radioactive probes. A ten times concentrate was prepared as follows;

Tris-HCl	60	mM
Magnesium chloride	100	mM
Mercaptoethanol	100	mM
pH	7.6	

2.2.14 *Microscopy*

a. Observation of infection threads: The methylene blue staining method (Vasse and Truchet, 1984) was used for a quick observation of infection threads in the intact root hairs. Plants were rinsed with sterile N-free F medium and then stained with 0.01% methylene blue (W/V) in filtered N-free F medium. Roots were stained for 10-15 minutes in a beaker at room temperature and then destained

for 2 hours with three changes of F medium. The stained plants were observed by bright-field microscopy.

b. Light microscopy: Specimens for light microscopy were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 25 mM phosphate buffer (pH 6.8) for 6 hours, post-fixed (after 3 rinses in buffer) in osmium tetroxide in distilled water for 2 hours, rinsed and dehydrated in a gradual acetone series. They were then embedded in Spurr's resin gradually over 3 days and polymerised in fresh resin overnight at 60°C. Sections were cut with a glass knife at 0.5 µm, stained with toluidine blue (pH 11.1), and viewed with Nikon Optiphot and Planapo objectives.

Table 2.1 Bacterial strains used

Strain	Relevant characteristics	Source or Reference
Fast-growing cowpea *Rhizobium*		
ANU240	Sm^r derivative of strain NGR234.	Morrison, 1984
ANU280	Rif^r derivative of ANU240.	This work
ANU265	Sp^r, Sym plasmid-cured derivative of NGR234.	Morrison, 1984
ANU266	Tn*5*-induced Exo^- mutant of ANU265	This work
1225	Tn*5*-induced Nod^- mutant of NGR234, Tn*5* inserted into the *nodD* gene on the Sym plasmid.	Morrison, 1984
R. trifolii		
ANU843	SU843, Nod^+Fix^+ on clovers. (originally J.M. Vincet collection)	Djordjevic *et. al.*, 1983
ANU845	Sym plasmid-cured derivative of ANU843, Sp^r.	Djordjevic *et. al.*, 1983
R. leguminosarum		
6015	Nod^-, Sym plasmid-deleted, *phe*-1, *trp*-12, *rif*-392, *str*-37.	Johnston *et. al.*, 1978
R. meliloti		
1021	Sp^r derivative of strain 2011, Nod^+Fix^+ on alfalfa	Meade *et. al.*, 1982

Table 2.1 continued

Fast-growing *Rhizobium japonicum*		
USDA191	Wild-type, Nod^+Fix^+ on soybean	Appelbaum *et. al.*, 1985
Parasponia Bradyrhizobium		
CP279	Nod^+Fix^+ on *Parasponia. andersonii*	Trinick and Galbrath, 1980
Agrobacterium tumefaciens		
C58	Virulent strain.	J. Schell
A136	Avirulent Ti plasmid-cured derivative of C58.	P. Hooykaas
E.coli		
HB101	F^-, *hsd*S20 (r^-, m^-), *ara*-14, *pro*A2, *gal*K2, *rps*L20, *xyl*-5, *mtl*-1, *sup*E44, *lac*Y1, *rec*A13, λ^-.	Bolivar *et. al.*, 1977
RR1	A rec^+ derivative of HB101.	Bolivar *et. al.*, 1977
HB1011	A Rif^r derivative of HB101.	This work
SM10	RP4::Mu integrated in the chromosome. Can mobilise plasmids *in trans*. Ap^r Tc^r Km^r.	Simon *et. al.*, 1983

Table 2.2 Plasmids used in this thesis

plasmid	Characteristics	Source or Reference
pSUP1011	pSUP101::Tn*5* suicide mutagenesis vector, Cm^r Km^r. Can be mobilised *in trans* like pSUP101. Cannot replicate in *Rhizobium* strain.	Simon *et. al.*, 1982
pBR322	Cloning vector, Ap^r Tc^r.	Bolivar *et. al.*, 1977
R68.45 Km^s	Km^s derivative of plasmid R68.45, Tra^+, Tc^r Ap^r.	Brewin *et. al.*, 1980
pMN2	Km^s derivative of pMO61, Cma^+, Tc^r Cb^r Ap^r.	Nayudu and Rolfe, 1987
pKan2	Specific Tn*5* probe containing internal 3.5kb *Hin*dIII fragment cloned into pBR322.	Scott *et. al.*, 1982
pE2811	The 6.3kb *Eco*RI fragment containing the Tn*5* flanking *Rhizobium* DNA of Exo^- mutant ANU2811 cloned into pBR322.	This work
pJG11	Recombinant plasmid of pUC18 containing a 10kb *Bam*HI subclone from plasmid R'3222, Ap^r	J.X. Gray
pSD1	The 6.2kb *Eco*RI fragment encompassing the 2861::Tn*5* insertion site cloned into pWB5a	Djordjevic S. 1987

Table 2.3 Plant species and cultivars

Plant species	Common name and cultivar
Macroptilium atropurpureum	cv. Siratro
Lablab purpureus	Lablab cv. Highworth
Desmodium intortum	Greenleaf desmo
Desmodium uncinatum	Silverleaf desmo
Leucaena leucocephala	cv. Peru
Parasponia andersonii	

CHAPTER THREE

ISOLATION AND CHARACTERISATION OF EXOPOLYSACCHARIDE-SYNTHESIS MUTANTS OF STRAIN NGR234

3.1 INTRODUCTION

Rhizobium strains involved in symbiotic association with leguminous plants often produce large amounts of exopolysaccharides (EPS). The role of EPS in plant-*Rhizobium* symbiosis has been investigated for many years (Bauer 1981; Rolfe and Shine, 1984). One approach has been the isolation of non-mucoid (Muc^-) exopolysaccharide-deficient mutants (Napoli and Albersheim 1980; Sanders *et. al.*, 1981). Studies with genetically-defined Muc^- mutants of *Rhizobium trifolii* strain have suggested that EPS, although not essential for nodule formation (Nod^+), is required for the establishment of an effective nitrogen-fixing nodule (Chakravorty *et. al.*, 1982).

Mutagenesis using transposon Tn*5* was successfully applied recently to the broad host-range fast-growing cowpea *Rhizobium* strain NGR234 (Morrison *et. al.*, 1983; Rolfe *et. al.*, 1983). A variety of mutant types were isolated including symbiotically defective strains. Several Tn*5*-induced symbiotic mutants were found on the Sym plasmid while others were mapped on the chromosome of strain NGR234 (Rolfe *et. al.*, 1983).

The data presented in this chapter show: (a) that the transposon mutagenesis system can be used to isolate a variety of mucoid-

defective mutants in strain NGR234; (b) that the effective nodulation (Nod^+Fix^+ phenotype) of these mutants may be a function of either the amount of EPS made, or the quality of the EPS made, or as a function of the test plant used; (c) that the plants producing different nodule types, either spherical (determinate) or meristematic (indeterminate) have different responses to various mucoid mutants.

3.2 ISOLATION OF TN5-INDUCED MUCOID DEFECTIVE MUTANTS

Strain ANU280 (a Sm^r, Rif^r derivative of strain NGR234) when plated out for single colonies on a variety of media forms homogeneous colonies. After Tn5 mutagenesis of strain ANU280 colonies with altered morphologies arose at a frequency of about 10^{-8} per recipient cell. Approximately 90 mutant strains were selected for a more detailed analysis by examination of their colony morphologies on Y agar medium containing different carbon sources. In this way mutants were classified into nine groups (Table 3.1). The different colony types ranged from non-mucoid (rough, Muc^-), semi-rough, crenulate, conditional mucoid to translucent (overproducers of EPS) colonies (Figs. 3.1, 3.2). The large number (39) of Group 2 (Muc^-) strains probably reflects the greater ease of recognizing this type of mutant on the selection plates.

3.3 NODULATION CHARACTERISTICS OF MUCOID-DEFECTIVE MUTANTS

Strain ANU280 can produce effective nodules (Nod^+Fix^+) on tropical legumes which form either spherical (determinate) or meristematic (indeterminate) nodules. Thus, the various mucoid-defective mutants

were tested on different host plants to examine whether alterations to the bacterial EPS has any effect on nodule formation. Criteria used to gauge a nodulation response to various mucoid-defective mutants were as follows: whether a nodule could be formed (Nod^+ verses Nod^-); whether nodule initiation was slow or normal rate of onset; whether nodule development was poor and whether nitrogen fixation occurred as judged by acetylene reduction assays. Comparisons were always made relative to the nodulation response shown by parent strain ANU280. Some of the different plant responses are shown in Fig 3.3 while Tables 3.2 and 3.3 record the various plant responses observed when inoculated with the different mutant groups. These results can be summarized as follows:

a) Most mutants which produced either slow initiating (Noi, S) or poor developing (Nod, P) nodules were unable to fix nitrogen (Nod^+Fix^-) on plants producing determinate nodules;

b) many rough mutants caused the formation of small callus-like structures on the roots of *Leucaena* plants (indeterminate nodule producer). These were phenotypically Fix^-;

c) translucent colony-forming mutants were usually slow to form nodules which were poorly developed and incapable of nitrogen fixation;

d) some mutants were Nod^+Fix^+ on some legumes but were Nod^+Fix^- on others.

The legumes which produced determinate nodules could not be used to predict the nodulation response of mucoid-defective strains. However, Muc^- (unconditionally rough) colony forming mutants formed only poorly developed nodules or callus-like structures on the roots of the indeterminate nodule-producing legume *Leucaena leucocephala*.

3.4 CHARACTERISATION OF MUC, *ADE* MUTANTS

The two mutants ANU2861 and ANU2866 over-produce EPS (Muc o/p) and form very mucoid, slimy colonies on mannitol media. Characterization of these strains showed them to be Muc o/p, Km^r (Tn*5*), Nod^- on siratro and *Desmodium uncinatum*, and to contain a single copy of Tn*5* inserted into different sized *Eco*RI fragments (Table 3.5). Both of these mutants were also ade^-. The isolation of ade^+ revertants of mutant ANU2866 produced strains that were Km^s Muc^+ (normal), Nod^+ on all test legumes and with no Tn*5* transposon. Mutant ANU2861 produced ade^+ revertants at low frequency and these were Km^r, Muc^+ (normal), Nod^+ on all test plants. Molecular analysis of these mutant ANU2861 revertants showed that Tn*5* was still present but transposed to a different location on the genome. Minimal media containing high concentrations of adenine supported growth of both ANU2861 and ANU2866, but at a reduced growth rate and level compared with the parent strain.

To determine whether the *ade* auxotroph was responsible solely for the observed symbiotic phenotype, plant nodulation assays were done on F plates containing varying concentrations of added adenine. Plants inoculated with mutants ANU2861 and ANU2866 were still Nod^-. Similarly, the Muc o/p phenotype could not be corrected by the addition of exogenous adenine. Interestingly, mutant ANU2866 induces a Nod^+Fix^+ phenotype on *Lablab purpureus* while mutant ANU2861 induces a Nod^+Fix^- phenotype on the plants that it can still nodulate (*Lablab* and *Leucaena*). Thus both mutants now possess a narrower host range.

From microscopic observations strains ANU2861 and ANU2866 were able to induce root hair curling but unable to induce the formation of

infection threads on siratro plants. A more detailed analysis of the Nod$^-$ phenotype of strain ANU2861 on siratro has been done by Ridge (1985). Some of his results are included in this thesis by permission and are only presented for clarity. These include three Figures (Figs. 3.4-3.6). Figure 3.4 shows that in curled hairs fixed 3 days after inoculation, the *Rhizobium* has almost penetrated the hair wall. The cell cytoplasm appears to be degenerated. There is a concentration of osmiophilic droplets at the penetration site, mainly at the plasma membrane. Osmiophilic droplets occur throughout the infected hair to a lesser extent. This is typical of strain ANU2861 infections. This type of reaction is very similar to a plant response to pathogenic invasion (Dickinson and Lucas, 1982).

In Figure 3.5, evidence of cortical cell division can be seen directly below the curled root hair. Recent studies (Bauer *et. al.*, 1985) have shown that *Rhizobium japonicum* is able to stimulate cell division in soybean plants without infection. Serial sections through several siratro examples proved there were no infection sites (Ridge, 1985). Figure 3.6a is a transmission electron micrograph showing dividing cortical tissue, stimulated by the presence of strain ANU2861. The epidermal cells shown in Fig. 3.6a appear to be necrotic, their cytoplasm and nucleus is considerably increased in electron density when compared to the tissue below the epidermal cells. However, the number of cells and the prominence of the nucleus and nucleolus in these necrotic cells indicates that they had undergone cell division prior to commencement of cell death. Figure 3.6a shows a band of OPD along the epidermal wall. High magnification of the epidermis (Fig. 3.6b) shows OPD concentrated in the cell walls and along the plasmalemma. No bacterial invasion of the tissue was detected through serial sections of five samples (Ridge, 1985).

3.5 POLYSACCHARIDE SYNTHESIS BY MUCOID-DEFECTIVE MUTANTS

Representatives of each mucoid-defective mutant group were analysed at different growth stages to determine the amount of exopolysaccharide (EPS) available. The findings (Table 3.4) can be summarized as follows:

a) The parent strain ANU280 increased its total excreted polysaccharides by 5 fold between mid and late logarithmic phases and by 6 fold between late logarithmic and stationary phase;

b) mutants ANU2849 and ANU2885 form similar colony morphologies and were both classified as Group 1 mutants but form very different levels of total polysaccharides. Mutant ANU2849 showed an almost 13 fold increase in EPS between late logarithmic and stationary phase while mutant ANU2885 showed only a 2 fold increase of EPS over the same growth period and formed only about 3% of wild-type levels in stationary phase. While mutant ANU2849 forms $Nod^{+}Fix^{+}$ nodules on *Leucaena*, mutant strain ANU2885 forms small calli on this plant.

c) different representatives of Groups 2 and 3 mutants produced similar reduced levels of EPS. The very rough mutants of these groups formed less than 10% of wild-type levels of EPS in stationary phase. These rough and semi-rough mutants fail to show a marked increase of EPS synthesis between late logarithmic and stationary phases. While the amounts of EPS produced by these two groups are similar, they show differences in their symbiotic responses. The Group 3 mutants form nodules on *Leucaena* whereas Group 2 mutants form calli.

d) Groups 4 and 5 mutants show a marked increase in their EPS synthesis between late log and stationary phases. Mutant ANU2814, which increases its EPS by 17 fold during this growth period, forms about 70% of wild-type levels of EPS and yet still forms only calli structures on the roots of *Leucaena* plants;

e) Group 9 mutants over-produce EPS at all tested growth stages forming up to 220% more EPS in stationary phase compared to the parent strain ANU280. Generally, members of this group form Fix^- nodules on test plants.

3.6 LOCATION OF TRANSPOSON TN5

To examine the molecular environment of Tn5 insertion site, total DNA was isolated from each mutant and digested separately with endonucleases *Eco*RI or *Cla*I or *Hin*dIII. Digested DNA was run on agarose gels and subsequently hybridized with radioactively-labelled Tn*5* DNA. Examination of autoradiographs of nitrocellulose filters containing *Eco*RI or *Cla*I digests of various mutants showed that Tn*5* had inserted, in most cases, into different sized DNA fragments (Table 3.5). Corresponding experiments using *Hin*dIII instead of *Eco*RI or *Cla*I was able to further discriminate between mutants where Tn*5* had inserted into similar sized *Eco*RI of *Cla*I fragments (Fig. 3.7, Table 3.5). Several mutants did retain two copies of the transposon (Table 3.5). A representative group of mutants were analysed on electrophoresis gels which separated plasmids from chromosomal material, and the DNA on these gels was subsequently hybridized with a Tn*5* probe. The Tn*5* probe hybridized to the Sym plasmid of mutant 1255 which has a Tn*5* inserted into the nodulation region of the Sym plasmid

of strain NGR234 (Morrison, 1984). In all the Muc$^-$ mutants examined, the Tn*5* probe did not hybridize to the Sym or Mega plasmid of any mutant strain (Fig. 3.8).

3.7 DISCUSSION

The experiments reported here show that transposon mutagenesis can be successfully applied to a fast-growing broad host-range cowpea *Rhizobium* to generate a variety of mutant strains with defective synthesis of surface polysaccharides. These studies have used a combination of tropical legumes which form either determinate or indeterminate type nodules to assess the consequences of altered cell surface carbohydrates on both nodule initiation and development. Generally, these alterations to surface polysaccharides do not block *Rhizobium* infection and nodule initiation but can have profound effects on the development of a functional nodule. On plants which normally produce spherical-type nodules the most extreme effect of the Muc$^-$ stains was to induce the production of small, non-nitrogen-fixing (Nod$^+$Fix$^-$) nodules. In contrast, on plants which produce indeterminate nodules, the most extreme effect of the Muc$^-$ strains was to induce small callus- or tumor-like structures, which were also Fix$^-$. There was no simple correlation between the amount of EPS produced and the symbiotic phenotype which resulted on plants. Extremely defective nodule development was found with mutants which had either markedly reduced EPS levels or with strains that had retained about 70% of parental strain polysaccharide levels or with mutants which over-produced exopolysaccharides. Thus, the effective (Fix$^+$) nodulation of a legume by a mutant defective in its polysaccharide

synthesis may be a function of either the amount of EPS formed or the nature of the EPS made.

Parent strain ANU280 is normally able to effectively nodulate all five test plants. By altering the polysaccharide synthesis of a strain it is possible to change the effective (Fix^+) host range of a *Rhizobium* strain. This can range from being unable to form effective nodules on any plant to losing the ability to form nitrogen-fixing nodules on only a single plant species.

Two particular mutants ANU2861 and ANU2866 (Group 9, mucoid translucent strains which also adenine auxotrophs) have partially lost their ability to initiate nodules on some test plants and have a more restricted host range. Genetic and physiological studies on these strains suggest that this restricted host range was not due to their adenine requirement but their defective polysaccharide synthesis. Possibly, the Tn5 insertion in each strain has inserted into an adenine biosynthetic gene(s) and has exerted a polar effect on a linked polysaccharide regulation gene(s) or *vice versa*. A detailed analysis of the Nod^- phenotype on siratro plants showed that mutant strain ANU2861 caused root hair curling (Hac^+ phenotype) and appeared to degrade the root hair at a region in the curled hair where penetration would normally occur with the parent strain NGR234 (Ridge and Rolfe, 1986). However, penetration of root hairs did not occur and the mutant strain failed to pass through the wall to the plasma membrane. As a result, mutant strain ANU2861 failed to induce an infection thread (Inf^- phenotype). A careful examination of electron micrographs of the root hairs showed that strain ANU2861 elicits a rejection-like response from the plant, in the form of osmiophilic droplets (OPD), which is a response similar to the hypersensitive

reaction induced by avirulent strains of *Pseudomonas solanacearum* (Sequeira *et. al.*, 1977). The OPD appear in the region of the penetration and at low concentration in the cell generally. These OPD are possibly phenolic compounds or phytoalexins that are commonly induced in pathogenic/plant relationships (Ingram *et. al.*, 1976; Miller and Maxwell, 1984). Stimulation of cortical cell division, induced by mutant strain ANU2861 without any obvious indication of entry into a root hair and with no infection thread formation, suggests that the bacterial signals responsible can be transmitted into the root cortex without invasion. There were OPD present in these cells next to the root surface, and necrosis of sub-epidermal cells (Ridge, 1985) suggests a response similar to the hypersensitive reaction (Doke *et. al.*, 1982; Klement, 1982; Tomiyama, 1982) although such necrosis may be the result of crushing pressure from cortical cell division. These essentially pathogenic responses by the plant are interesting in view of the hypothesis that the *Rhizobium*-legume symbiosis is a "controlled disease" (Rolfe *et. al.*, 1982; Vance, 1983).

Mapping studies have indicated that all the Muc$^-$ mutants so far tested are probably located on the bacterial chromosome since hybridization to plasmid DNA from these mutant strains did not show a positive response. In addition, it has been possible to co-transfer the Tn*5* mutation and its concomitant Muc$^-$ phenotype for several mutants, such as strain ANU2811, into strain ANU265 (a Sym plasmid-cured derivative of strain NGR234) using a kanamycin-sensitive derivative of plasmid R68.45 (see Chapter 4). This finding plus the analysis of occasional revertants that have arisen for some mutants enables the following conclusion to be made: that the phenotype of

most of the Muc$^-$ mutants arose from the single insertion of a Tn*5* transposon. Probably the most important result is that a broad host range bacterium such as NGR234 can be evolved by mutation into a strain with a narrower host range which is able to induce "hypersensitive reaction" on certain host plants.

3.8 ACKNOWLEDGEMENT

Some of the results shown in this chapter have appeared in the paper "Alteration of effective nodulation properties of a fast-growing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis" by Hancai Chen, Michael Batley, John Redmond and Barry G. Rolfe in Journal of Plant Physiology, volume 120: 331-349 (1985).

Table 3.1 Colony morphology of Muc$^-$:Tn5 mutants of ANU280 growing on various carbon sources

Mucoid group		Colony morphology						
		Mannitol (39 gl^{-1})	Mannitol (5 gl^{-1})	Glucose	Sucrose	Sorbitol	Mannose	Fructose
Parent	ANU280	mucoid	mucoid	mucoid	mucoid	mucoid	mucoid	mucoid
1	(14)	mucoid crenate	semi-rough crenate	rough crenate	rough crenate	rough crenate	rough crenate	rough crenate
2	(39)	rough	rough	rough	rough	rough	rough	rough
3	(4)	semi-rough SC	semi-rough LC	semi-rough SC	semi-rough LC	semi-rough SC	semi-rough SC	semi-rough SC
4	(2)	mucoid	semi-rough	rough	rough	rough	rough	rough
5	(3)	semi-rough	rough	rough	rough	rough	rough	rough
6	(3)	mucoid	mucoid	less mucoid	less mucoid	less mucoid	less mucoid	less mucoid
7	(7)	mucoid	semi-rough	semi-rough	semi-rough	semi-rough	semi-rough	semi-rough
8	(1)	mucoid	less mucoid	semi-rough LC	semi-rough SC	semi-rough SC	semi-rough LC	semi-rough LC
9	(5)	mucoid translucent	mucoid translucent	mucoid lesss translucent	mucoid less translucent	mucoid less translucent	mucoid less translucent	mucoid less translucent

Each purified colony was streaked on Y agar medium containing 5 gl^{-1} of carbon source and incubated at 30^{o}C for 4-6 days. SC, small colony; LC, large colony.

Table 3.2 Plant nodulation responses to various Muc$^-$ mutant classes.

Mucoid group (ANU strain)	Determinate nodule producer								Indeterminate nodule produer	
	Macroptilium atropurpureum		*Desmodium intortum*		*Desmodium uncinatum*		*Lablab purpureus*		*Leucaena leucocephala*	
	Noi[a])	Nod[b])	Noi	Nod	Noi	Nod	Noi	Nod	Noi	Nod
1. (rough or musoid crenate)										
2802	N	P	N	N	S	P	S	P	N	N
2803	N	N	S	P	N	P	S	P	N	N
2805	N	P	N	N	S	P	S	P	S	N
2816	N	N	S	P	S	P	S	N	S	P
2819	N	N	S	P	N	P	N	P	S	N
2828	N	N	N	N	N	N	N	N	S	N
2849	N	P	N	N	N	P	S	P	N	N
2856	S	N	N	N	S	P	N	N	S	P
2857	N	N	N	N	S	P	S	N	S	N
2883	N	N	N	N	S	P	S	N	S	N
2885	N	N	N	N	N	N	N	N	samll calli[c])	
3. (semi-rough, variable colony size)										
2809	N	P	N	P	N	N	S	P	N	N
2834	N	P	S	P	S	P	N	P	S	P
2835	N	P	S	P	S	P	S	P	S	P
2836	N	P	N	P	S	P	S	P	S	P
4. (variable mucoid)										
2801	N	P	S	P	S	P	S	P	small calli	
2825	N	N	N	N	S	P	S	P	small calli	

Table 3.2 Continued

5. (variable rough)										
	2814	N	N	S	P	S	N	N	P	small calii	
	2827	N	P	S	P	S	P	N	P	S	N
	2893	N	N	N	N	S	P	N	N	S	N
6. (slightly less mucoid)										
	2837	N	P	S	P	N	P	N	P	S	N
	2860	N	P	N	N	S	P	N	N	S	P
	2869	N	P	S	P	S	P	N	P	S	P
7. (mucoid on mannitol, semi-rough)										
	2812	N	P	S	P	S	P	N	N	N	N
	2821	N	N	S	P	S	P	S	P	S	N
	2859	S	N	N	P	S	P	S	N	N	P
	2899	S	P	S	P	S	P	S	P	S	P
8. (mucoid, semi-rough)										
	2858	S	P	S	P	S	P	S	P	S	P
9. (mucoid translucent)										
	2833	S	P	S	P	S	P	S	P	S	P
	2861	-		-		-		S	P	S	P
	2866	-		S	P	-		N	N	S	P
	2894	S	P	S	P	S	P	S	P	N	N
	2895	S	P	S	P	S	P	S	P	S	N

[a]) Noi, nodule initiation; N, normal nodule initiation; S, slow to form nodule; NT, not tested; -, Nod^-.

[b]) Nod, nodule development; N, normal nitrogen-fixing (Fix^+) nodules; P, poorly developed nodules (Fix^-).

[c]) Small callii, these are small defectively formed nodules which have a plant callus or tumor appearance.

Table 3.3 Plant nodulation responses to different Group 2 Muc⁻ mutants

Mucoid group (ANU strain)	Determinate nodule producer								Indeterminate nodule produer	
	Macroptilium atropurpureum		*Desmodium intortum*		*Desmodium uncinatum*		*Lablab purpureus*		*Leucaena leucocephala*	
	Noi	Nod	Noi	Nod	Noi	Nod	Noi	Nod	Noi	Nod
2. (unconditionally rough)										
2807	S	P	N	N	S	P	S	N	small calli	
2808	N	P	N	N	S	P	S	P	small calli	
2811	S	P	N	P	N	P	S	P	small calli	
2817	N	N	N	N	S	P	N	P	small calli	
2818	N	P	N	N	N	N	S	P	small calli	
2820	S	P	N	N	S	P	S	P	small calli	
2822	N	P	N	N	N	N	N	N	small calli	
2823	N	P	N	P	S	N	N	N	small calli	
2824	N	N	N	N	S	N	N	P	small calli	
2826	N	P	N	P	S	P	N	N	small calli	
2831	N	N	N	N	S	P	N	N	small calli	
2838	N	N	N	N	N	N	N	P	small calli	
2840	N	N	N	P	S	P	N	P	small calli	
2841	S	P	N	N	N	P	N	N	small calli	
2842	S	P	N	P	S	P	S	P	small calli	
2844	N	P	N	N	S	P	N	N	small calli	
2845	N	P	N	N	N	P	N	N	small calli	
2847	N	N	N	N	N	N	N	P	small calli	
2851	S	P	S	P	S	P	N	N	small calli	
2852	N	N	N	P	N	N	N	N	small calli	
2854	N	N	N	N	N	P	N	P	small calli	
2864	S	P	N	N	S	P	N	N	small calli	
2865	S	N	N	P	N	P	N	P	small calli	
2867	N	N	S	N	N	N	S	N	small calli	
2871	N	N	N	P	S	P	S	P	small calli	
2872	S	N	N	N	S	N	S	P	small calli	
2873	S	N	N	N	N	N	S	N	small calli	
2875	S	P	N	N	N	N	N	N	small calli	
2876	N	N	N	N	S	N	N	P	small calli	
2877	N	N	N	N	S	N	N	N	small calli	

The legend to Table 3.3 is as described for Table 3.2.

Table 3.4 EPS levels of parent strain ANU280 and different Muc⁻:Tn5 mutants

Strain	Mucoid group	Mid log phase		Late log phase		Stationary phase	
		mg hexose/ mg protein	mg uronic acid/ mg protein	mg hexose/ mg protein	mg uronic acid/ mg protein	mg hexose/ mg protein	mg uronic acid/ mg protein
ANU280	parent	0.09	0.17	0.47	0.37	3.0	3.0
ANU2849	1	0.15	0.20	0.16	0.28	2.2 (73)	2.1
ANU2885	1	0.02	0.15	0.03	0.13	0.09 (3)	0.1
ANU2807	2	0.04	0.17	0.05	0.14	0.18 (6)	0.11
ANU2811	2	0.04	0.15	0.07	0.13	0.19 (6)	0.12
ANU2909	3	0.06	0.20	0.07	0.08	0.14 (5)	0.08
ANU2834	3	0.07	0.21	0.08	0.08	0.28 (9)	0.14
ANU2801	4	0.05	0.23	0.07	0.11	0.70 (23)	0.31
ANU2814	5	0.11	0.15	0.12	0.20	2.1 (70)	1.8
ANU2827	5	0.16	0.19	0.17	0.28	2.1 (70)	1.5
ANU2860	6	0.15	0.34	0.23	0.28	2.3 (77)	1.6
ANU2869	6	0.27	0.27	0.30	0.26	1.1 (37)	0.58
ANU2812	7	0.20	0.23	0.38	0.42	1.4 (46)	0.98
ANU2899	7	0.10	0.25	0.12	0.21	0.41 (14)	0.41
ANU2858	8	0.10	0.41	0.07	0.22	0.30 (10)	0.39
ANU2894	9	0.75	1.0	2.7	2.22	6.6 (220)	6.7

All strains were grown in BMM medium.

The amount of polysaccharide is also expressed as a percentage of the parent strain and shown in brackets.

Exopolysaccharide synthesis mutants derived by transposon Tn*5* were initially characterised on BMM medium plates. *Rhizobium* cultures grown in BMM liquid medium are likely to differ from those prepared from BMM-agar. Oxygen tension and the availability of a firm surface for attachment are likely to play a role in the regulation of EPS synthesis.

Table 3.5 Copy number and location of Tn*5* of mocoid-defective mutants of strain ANU280

Mucoid group	Total DNA isolated strain (ANU)	Copy number of Tn*5*	^{32}P-labelled Tn*5* probe hybridized to the DNA fragmenet(s)* digested with				
			*Eco*RI	*Cla*I	*Hind*III		
1	2856 2857	1	12.8		19	8.5	3.5
1	2816	1	12.8		19	13	3.5
1	2805	1	9.0		19	10	3.5
1	2919	1	9.0		19	8.5	3.5
1	2828	1	10.4		19	10	3.5
1	2832	2	8, 20				
1	2883	1	17		5.5	1.4	3.5
1	2885	1	16		8.0	4.4	3.5
1	2803	1	16				
1	2802	1	23		4.8	1.4	3.5
1	2849	1	14				
2	2820 2826 2831	1	13	6.8			
2	2822 2824	1	7.5	15			
2	2871 2872 2873 2875 2876 2877	1	13	7.5	6.0	1.65	3.5
2	2867	1	13	7.8			
2	2808 2838 2840 2841 2842 2844 2845 2847	1	7.2	8.1	1.7	1.5	3.5
2	2823 2890	1	7.2	8.1	19	5.0	3.5
2	2807 2811 2851 2852 2854	1	6.4	8.1	5.0	1.3	3.5
2	2864 2865	1	6.4	8.1	1.96	1.3	3.5
2	2818	1	10.4	6.4	8.0	6.0	3.5
2	2817	1	15.1		17	7.0	3.5
2	2884	2	6.7, 30				
2	2863	2	15, 20				

Table 3.5 continued

Mucoid group	Total DNA isolated strain (ANU)	Copy number of Tn*5*	^{32}P-labelled Tn*5* probe hybridized to the DNA fragmenet(s) digested with			
			*Eco*RI	*Cla*I	*Hin*dIII	
3	2809	1	7.0	7.5	1.5	3.5
3	2834	1	8.5	1.7	1.2	3.5
3	2835	1	8.0	5.7	1.95	3.5
3	2936	1	8.5	5.4	1.95	3.5
4	2801	1	15	6.0	3.0	3.5
4	2825	1	14	8.5	4.4	3.5
5	2814	1	10	10	3.0	3.5
5	2827	1	6.7	5.5	1.8	3.5
5	2893	1	11.5	6.2	2.9	3.5
6	2950	2	7.0, 25			
6	2869	1	11.8	20	11	3.5
6	2860	1	9.4	9.0	5.0	3.5
6	2837	1	17.6	19	5.4	3.5
7	2812	1	13	11	3.5	3.5
7	2859	1	10	2.0	1.4	3.5
7	2899	1	17	8.8	3.0	3.5
7	2821	1	12	8.8	1.5	3.5
8	2858	1	6.7	9.0	7.0	3.5
9	2833	1	7.5	4.0	1.0	3.5
9	2861	1	15	3.8	1.5	3.5
9	2866	1	9.0	6.0	3.8	3.5
9	2894	1	15	3.0	3.0	3.5
9	2895	1	7.5	16	8.0	3.5

* The molecular weight of DNA fragments are indicated in kb. Plasmid pKan2 was used as the probe to hybridize to the DNA digested with *Eco*RI or *Cla*I. Plasmid pSUP1011 was used as the probe to hybridize to the DNA digested with *Hin*dIII. Tn*5* has one cleavage site of *Eco*RI or *Cla*I, and two clevage sites of *Hin*dIII. The 3.5 kb *Hin*dIII fragment is the internal Tn*5* sequence.

Fig. 3.1 Comparison of the colony morphologies of strain ANU280 and its derivatives. All strains were grown on Y agar medium containing mannitol. a) Parent strain ANU280, mucoid; b) mutant ANU2894, over-producer of exopolysaccharide, translucent; c) ANU2899, semi-rough; d) ANU2820, rough-dry colony morphology.

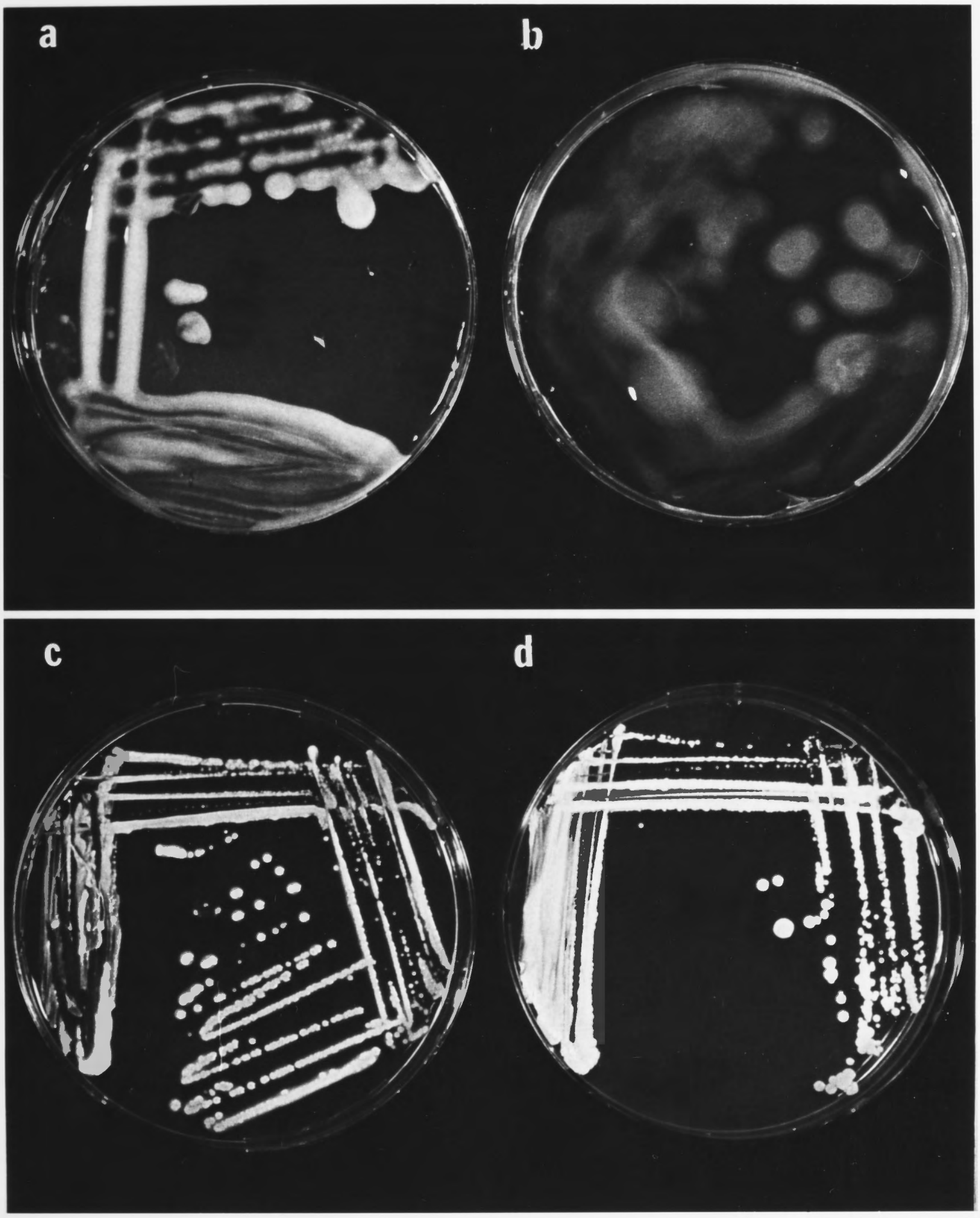

a
b
c
d

Fig. 3.2 Colony morphologies of conditional non-mucoid strains derived from strain ANU280. a) Mutant ANU2825 mucoid colonies on Y agar medium containing mannitol (39 g l^{-1}); b) ANU2825 rough colony morphology on Y agar containing mannitol at 5 g l^{-1}; c) mutant ANU2883 mucoid colonies on Y agar containing mannitol (39 g l^{-1}); d) mutant ANU2883 forms a rough-crenate colony morphology on Y agar containing mannitol at 5 g l^{-1}.

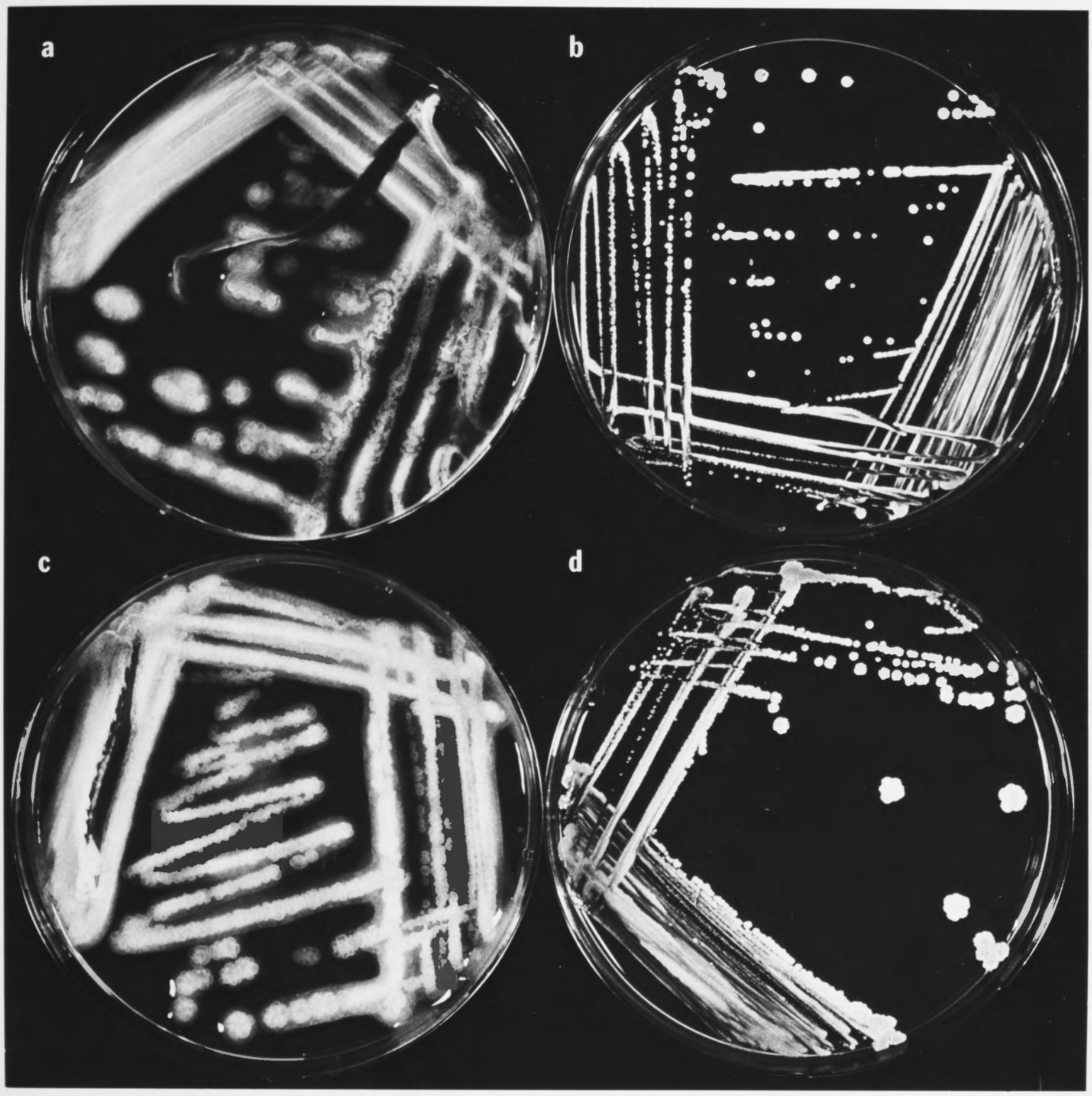
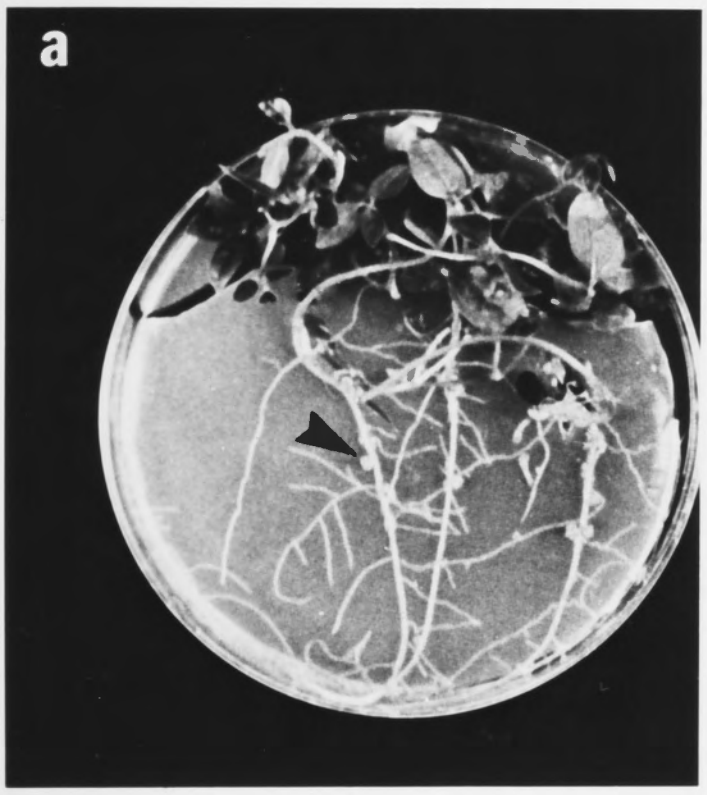

a
b
c
d

Fig. 3.3 Plant responses to inoculation with mucoid-defective mutants. a) Nod^+Fix^+ response of siratro inoculated with parent strain ANU280; b) siratro inoculated with mutant ANU2842 (Group 2) produces slow forming, small non-nitrogen-fixing nodules (Nod^+Fix^-); (c) *Leucaena leucocephala* inoculated with (left) strain ANU280 (Nod^+Fix^+), (right) mutant ANU2807 which forms a small callus structure, non-nitrogen-fixing; d) Nod^+Fix^+ (C_2H_2 reducing) nodule of *Leucaena* inoculated with strain ANU280; e) callus-like structure formed on *Leucaena* after inoculation with mutant ANU2807, non-nitrogen-fixing.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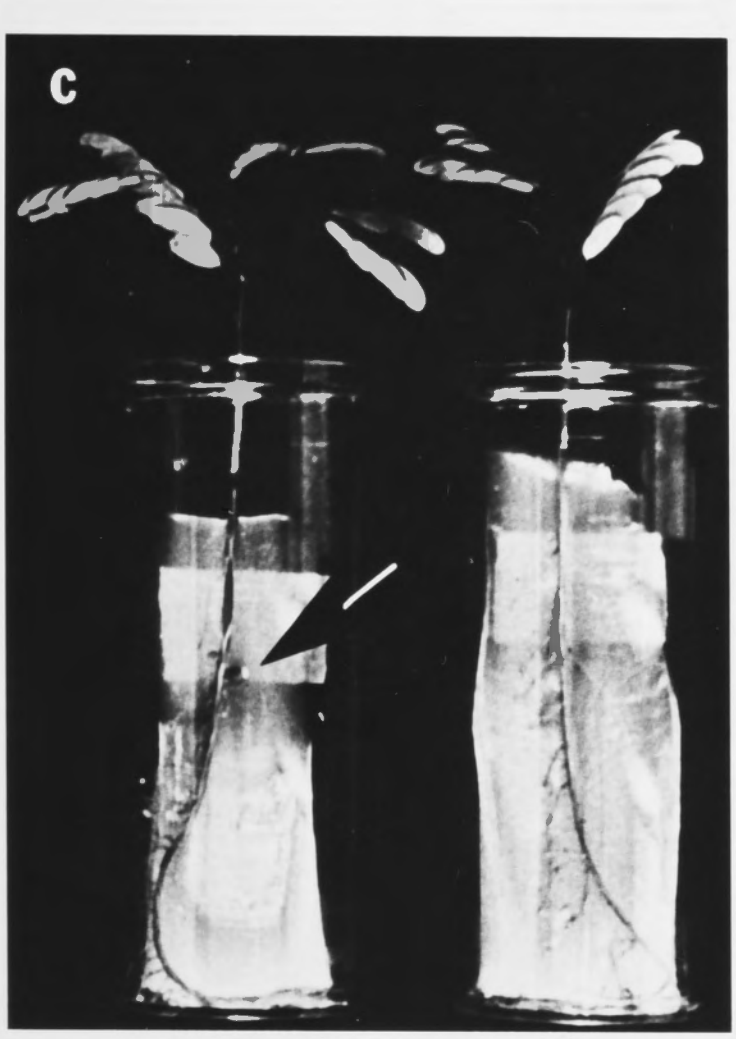
b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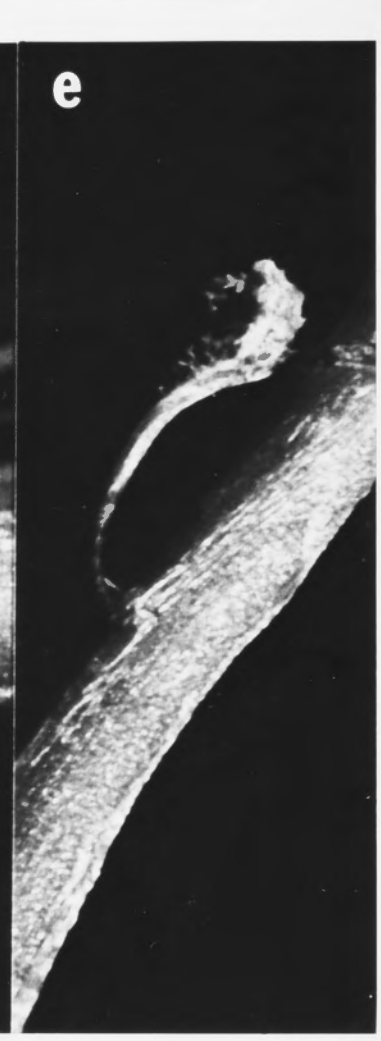
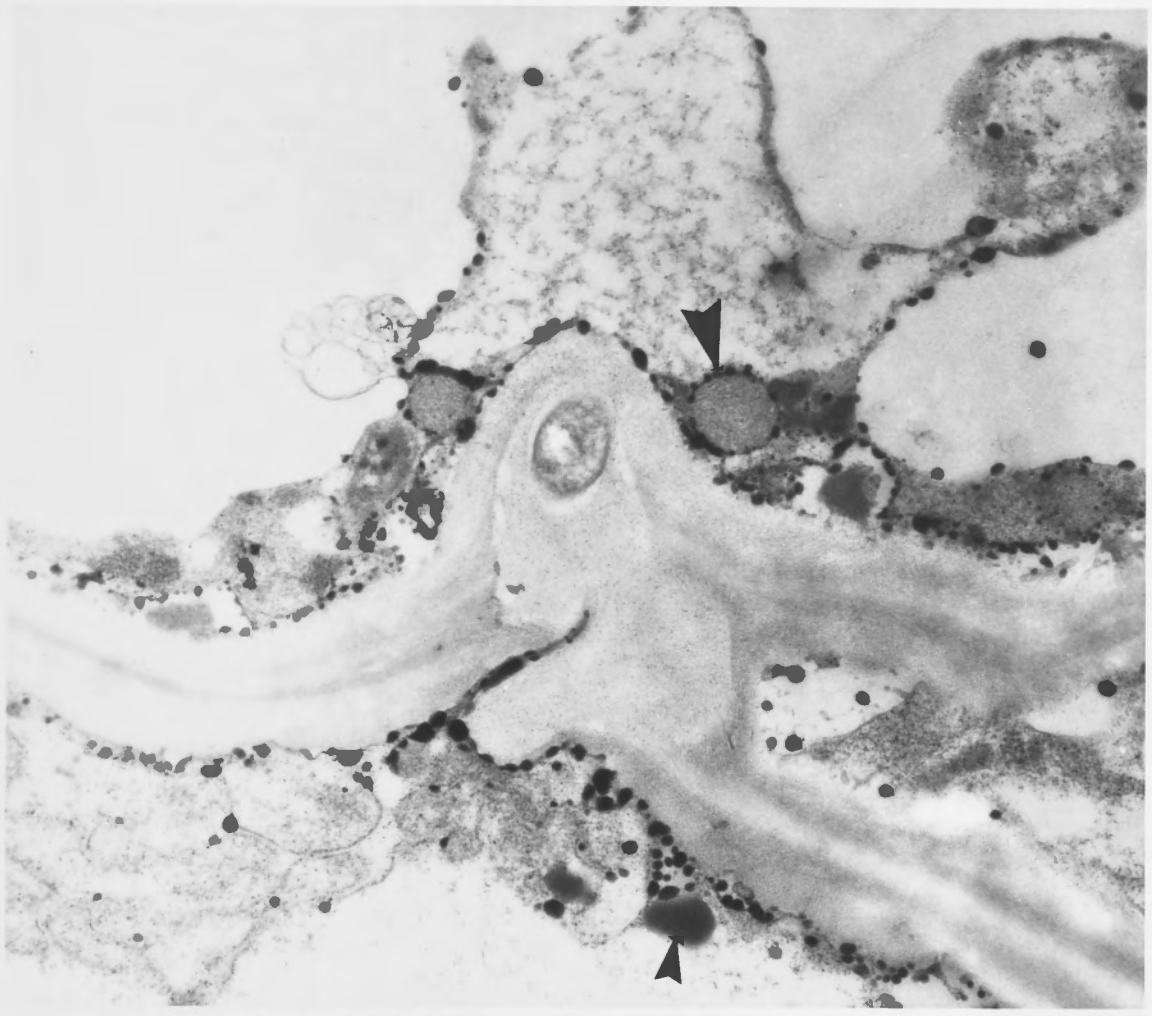

e

Fig. 3.4 Transmission electron micrograph showing the penetration site of mutant ANU2861 infection. Osmiophilic droplets (OPD) completely surround the penetration site in these sections, but not at the centre point of invasion (Ridge, 1985). This demonstrates that their accumulation at the site is similar to a truncated cone in shape, completely surrounding the penetration except at the very apex or penetration point. Osmiophilic droplets also accumulate around the mitochondria (large arrowhead). Less electron dense bodies (small arrowhead) are thought to be lipid material. (a) X20,000; bar = 1μm. (b) X34,000; bar = 1μm.



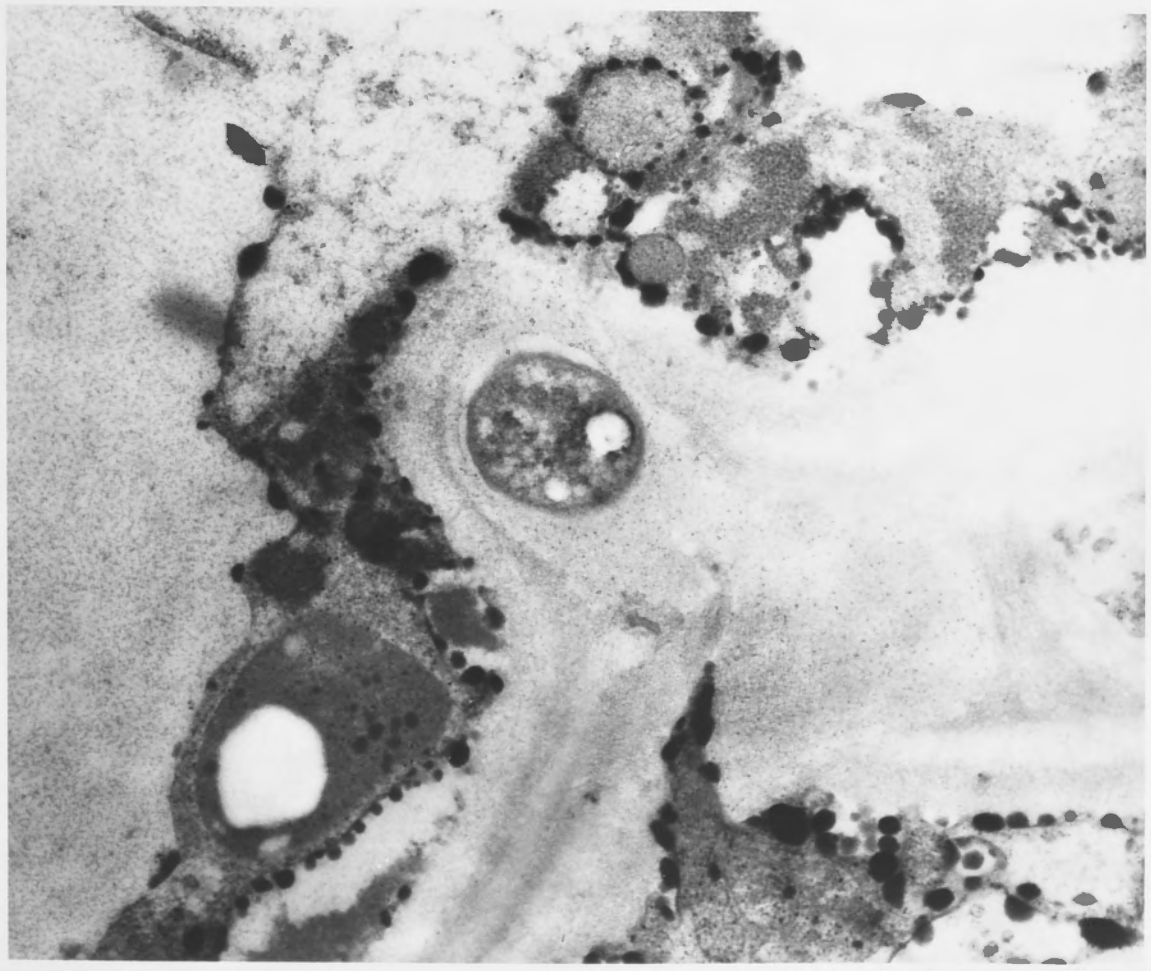


Fig. 3.5 Closer views of an example of a cell division area caused by strain ANU2861. The cell division is disorganized, there are no longitudinally-arranged files of cells that occur in strain NGR234 infection (Ridge, 1985). The infected root hair is indicated with an arrowhead. (a) X100; bar = 200µm. (b) X260; bar = 100 µm.

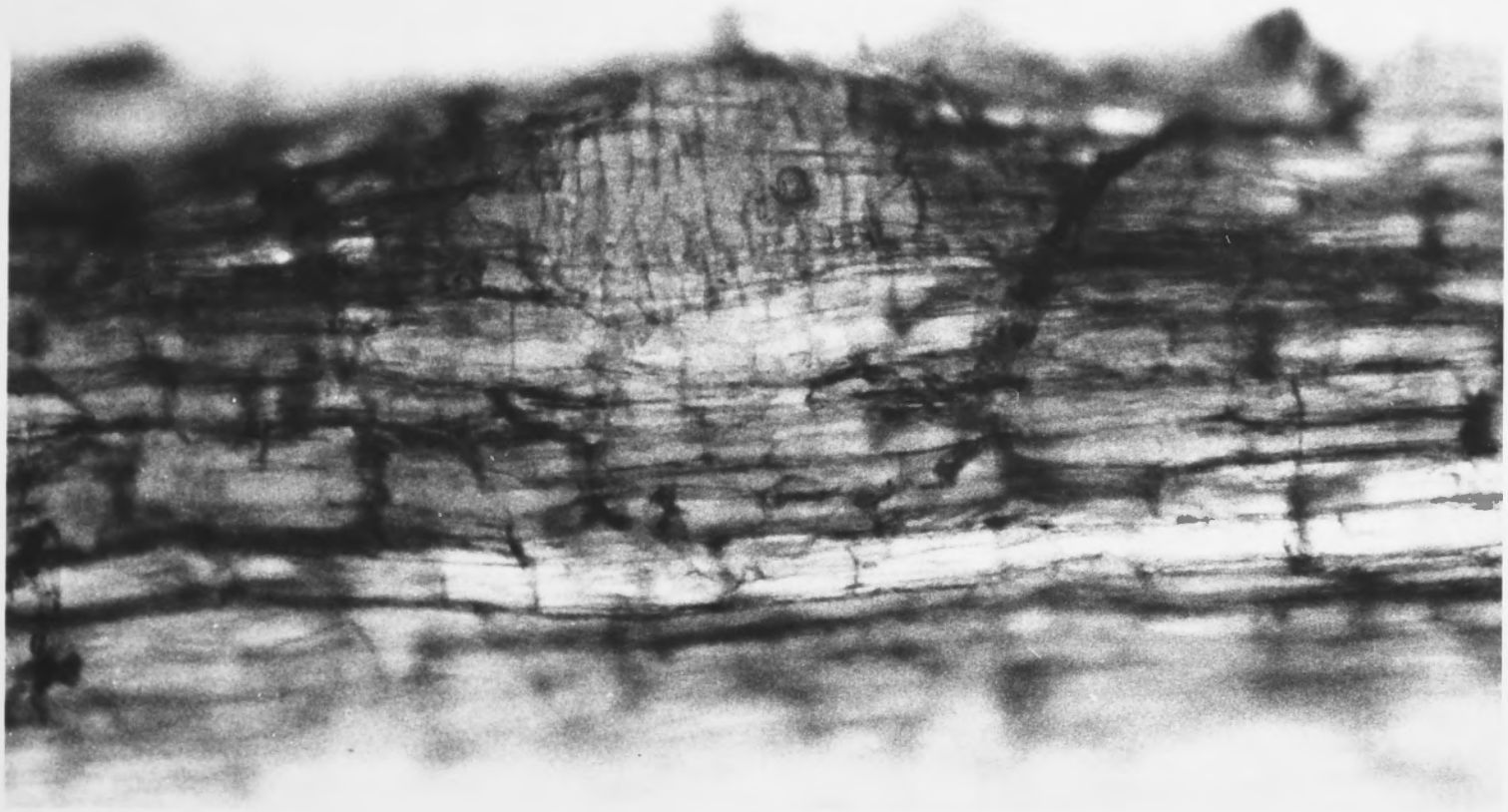
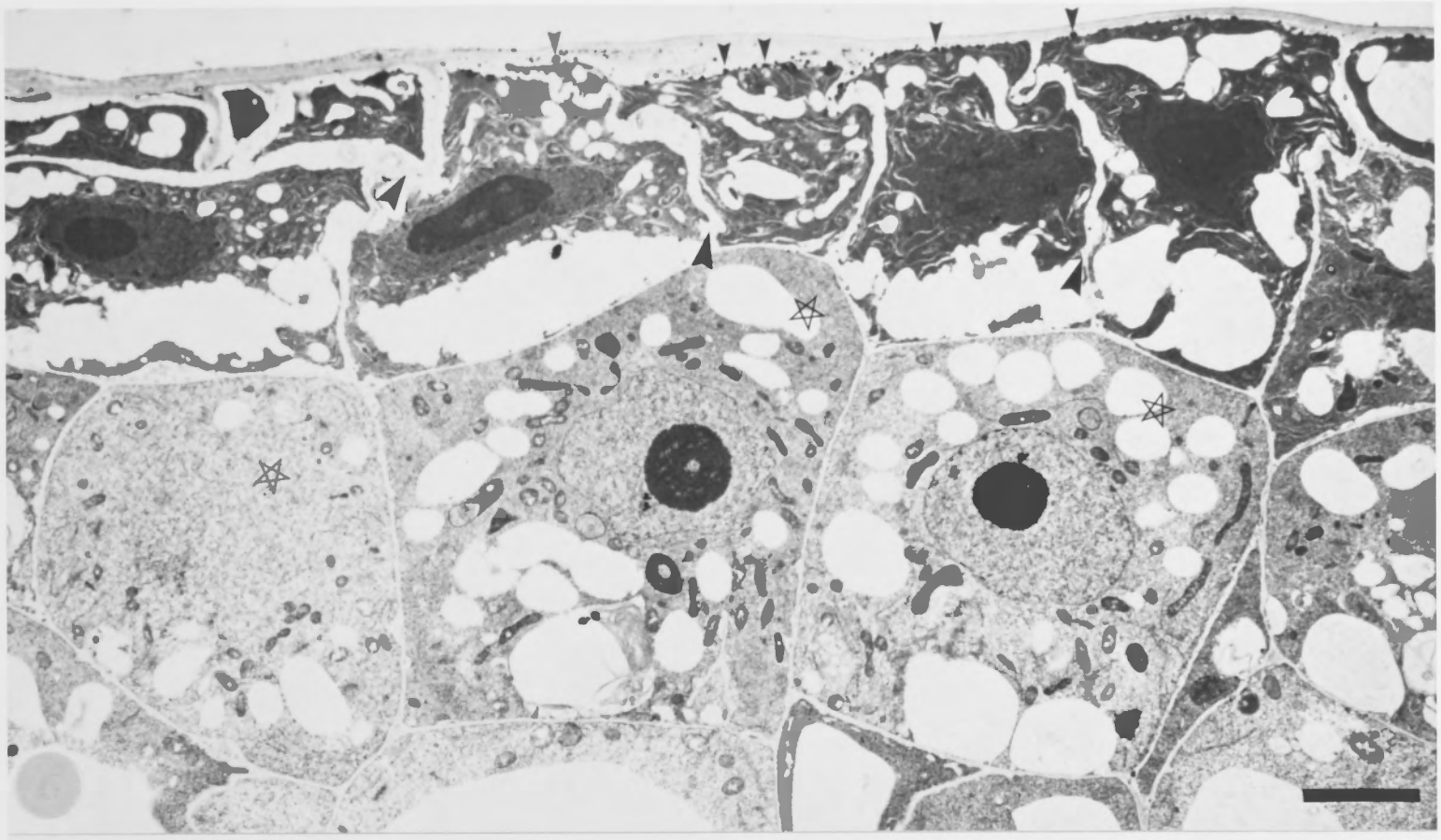


Fig. 3.6 (a) A low magnification transmission electron microscope view of the central part of a locus of cell division caused without invasion by mutant ANU2861. In this example the epidermal cells (uppermost cells in micrograph) appear to have undergone cell division but were in the process of necrosis at the time of fixation, judging by the densely staining nature of the cytoplasm. This is possibly due to the pressure of cortical cell division within the root. Certainly the epidermal walls are quite distorted (large arrowheads). Accumulation of OPD occurred mostly in the outer walls of the epidermis (small arrowheads). The cortical cells (stars) appear as normal (dividing) tissue. X3,750; bar = 4 μm.

(b) High magnification transmission electron microscope view of epidermal wall (small arrowheads indicate the root surface) of a section. OPD (large arrowheads) accumulated in the cell wall, mostly next to the plasma membrane. X70,000, bar = 200μm.



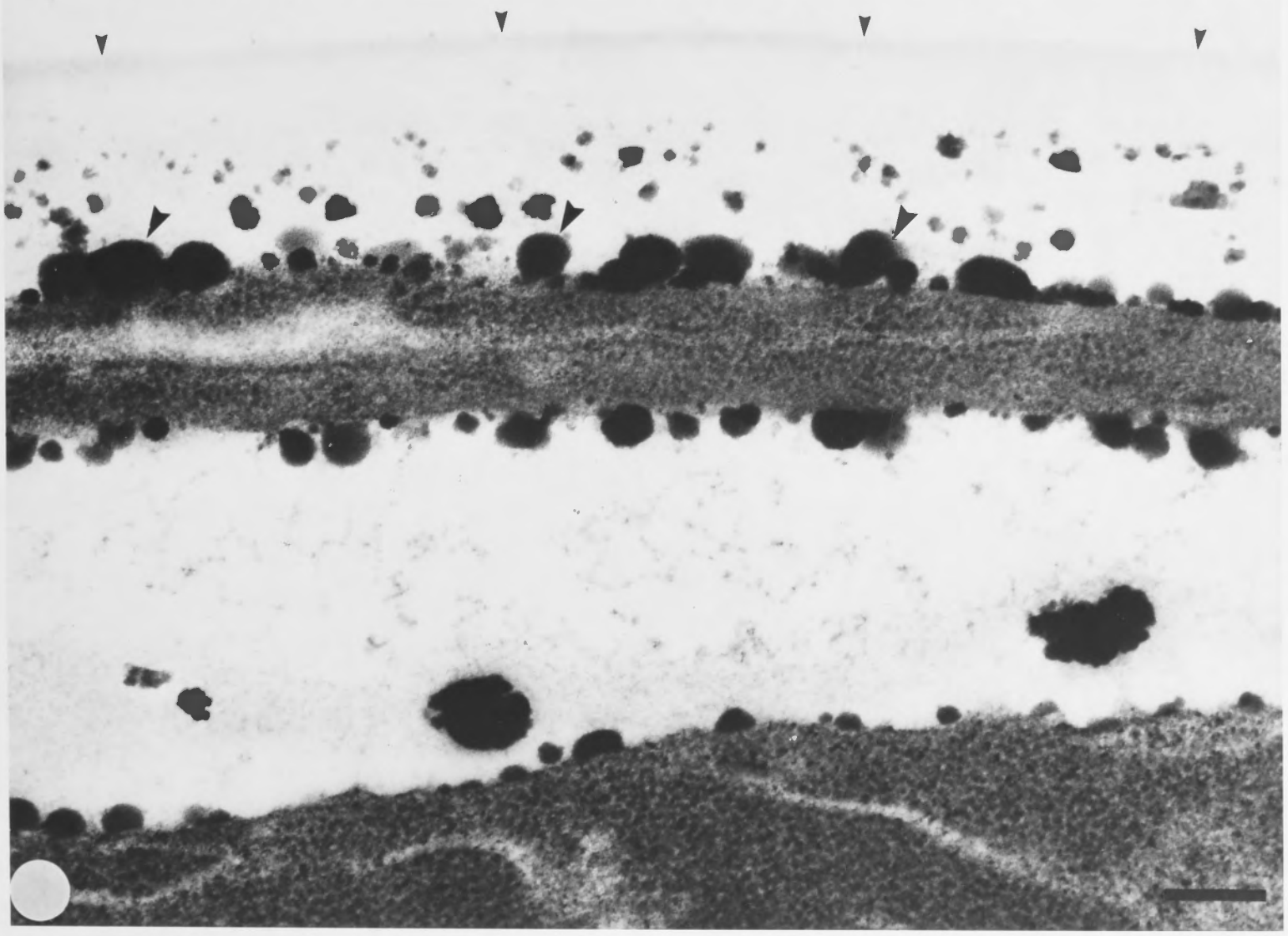


Fig. 3.7 Hybridization of a ^{32}P-labelled Tn*5* probe to total DNAs of various non-mucoid strains. Plasmid pSUP1011 which contains Tn*5*, was found to hybridize to different patterns of *Hin*dIII bands in each of the Tn*5*-induced non-mucoid mutants screened. Only the characteristic 3.2 kb *Hin*dIII internal Tn*5* sequence (arrow) was common. Thus Tn*5* had inserted into different locations in each of the mutants. Lane 1, *Hin*dIII digested λ DNA; 2, mutant ANU2865; 3, ANU2863; 4, ANU2825; 5, ANU2885; 6, ANU2899; 7, ANU2807; 8, ANU2808; 9, ANU2871; 10, ANU2826; 11, ANU2823; 12, ANU2842.

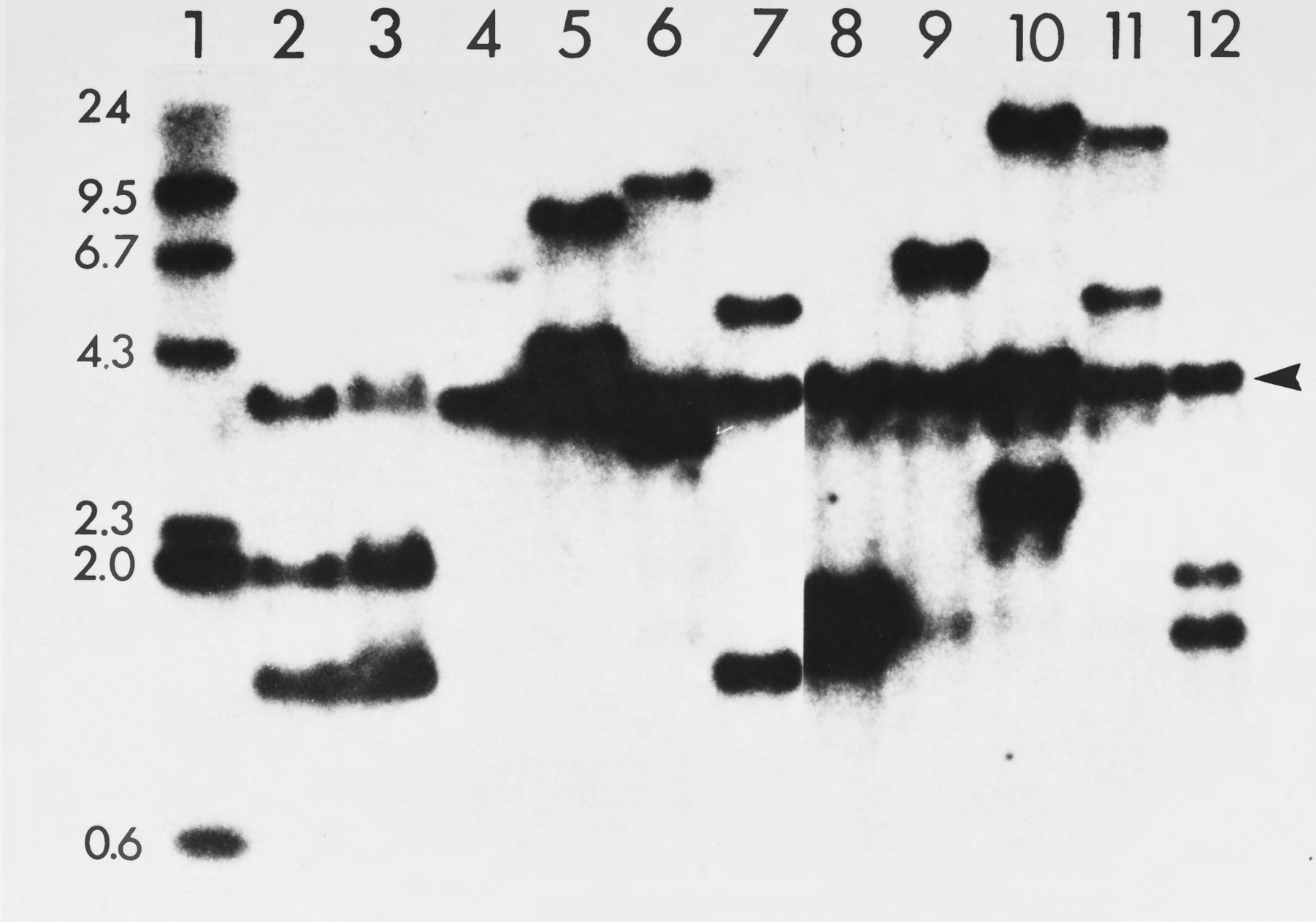
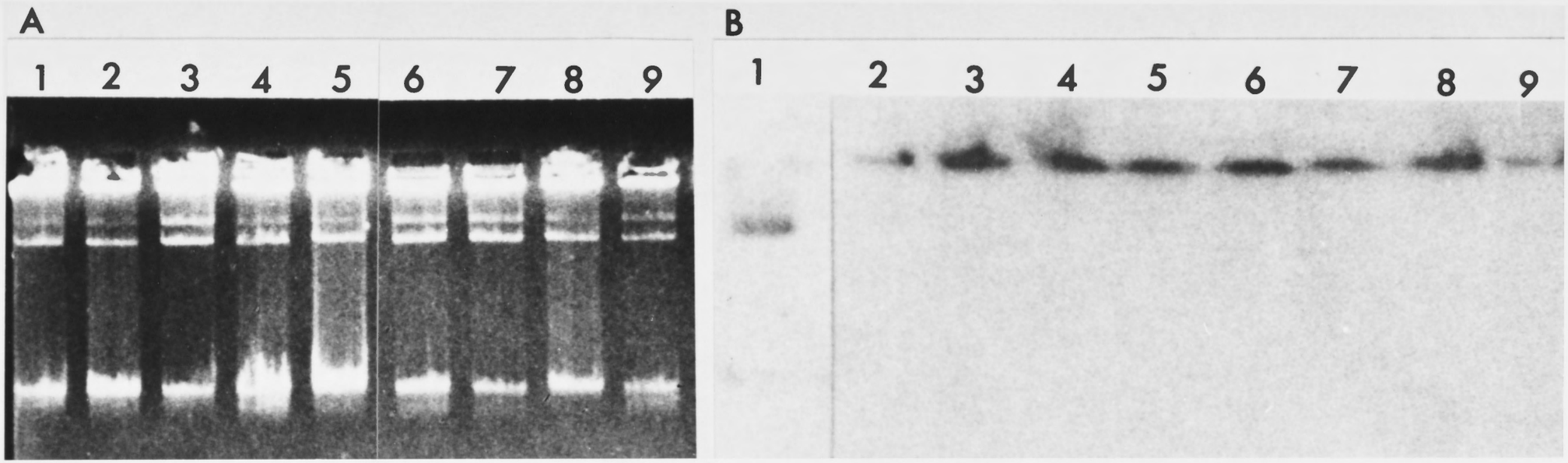

1 2 3 4 5 6 7 8 9 10 11 12
24
9.5
6.7
4.3
2.3
2.0
0.6

Fig. 3.8 Plasmid profiles of strain ANU280 and its mucoid-defective mutants (A). Lane 1, ANU1255; 2, ANU280; 3, ANU2801; 4, ANU2811; 5, ANU2820; 6, ANU2840; 7, ANU2833; 8, ANU2869; 9, ANU2858. The corresponding autoradiograph is shown in (B). Lane 1, control strain ANU1255 which contains a Tn*5* insertion on the Sym plasmid; lanes 2 to 9 contain different Tn*5*-induced mucoid-defective mutants of strain ANU280. A probe containing transposon Tn*5* sequences (pKan2) was used. Hybridization to plasmid species only occurred in lane 1.


A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9

CHAPTER FOUR

CELLULAR COMPLEMENTATION RESULTS IN EFFECTIVE NODULATION OF *LEUCAENA LEUCOCEPHALA*

4.1 INTRODUCTION

In chapter three, transposon Tn*5* mutagenesis was successfully applied to the fast-growing broad-host range cowpea *Rhizobium* strain ANU280 to generate nine groups of mutant strains with altered surface polysaccharide production. With unconditionally rough (Group 2) mutants these alterations did not block nodule initiation (Noi^+) on most plants but did have profound effects on the development of a functional nitrogen-fixing nodule. Different plants had different responses to individual EPS-deficient mutants and most of the mutants became "narrow host-range" with respect to the ability to induce a nitrogen-fixing response. In contrast to other tested legumes, the tropical legume *Leucaena leucocephala* (which produces an indeterminate nodule) was more sensitive in its response to the Group 2 Muc^- mutants (Table 3.3). These mutants, which produced the least amount of EPS and formed rough non-mucoid colonies (Fig. 3.1), induced the formation of callus-like structures instead of normal nitrogen-fixing nodules on *Leucaena* plants (Fig. 3.3). These group 2 Muc^- mutants are primarily examined in this chapter.

The possibility of exopolysaccharides being involved in the formation of nitrogen-fixing (Fix^+) nodules was supported by strain mixing experiments (Rolfe *et. al.*, 1980 b). These studies involved the mixing and coinoculation of two mutant strains (SU846 and SU847)

which were spontaneously derived from *Rhizobium trifolii* strain NA34 onto white clover plants. When strain SU846 ($Muc^-Nod^+Fix^-$) was mixed with strain SU847 (Muc^+Nod^-, Sym plasmid-deleted), a cooperative interaction between the $Muc^-Nod^+Fix^-$ and the Muc^+Nod^- mutant led to the formation of an effective (nitrogen-fixing) nodule on clover plants (Rolfe *et. al.*, 1980 b). It was suggested that invasive Muc^- strain were responsible for the formation of nodules and the entry of both strains into the root hair while the Nod^- strain produced the normal levels of EPS required for the establishment of functional nodules (Rolfe *et. al.*, 1980 b; 1981 b). In this chapter, the possible relationship between the establishment of effective (nitrogen-fixing) nodules and *Rhizobium* surface polysaccharides has been further investigated by inoculating the tropical legume *L. leucocephala* with mixtures of a $Muc^-Nod^+Fix^-$ (callus-forming) mutant and a Muc^+Nod^- (Sym plasmid-cured) mutant of strain NGR234.

4.2 NODULATION OF *LEUCAENA* BY THE WILD-TYPE STRAIN AND MUC^- MUTANTS

The parent strain ANU280 formed cylindrical, pink pigmented nodules on *Leucaena* plants which readily reduced acetylene. Sections through these nodules showed a meristematic zone with well formed vascular bundles, and an extensive bacteroid zone containing rod shaped non-swollen bacteria (Fig. 4.2). All Muc^- mutants used in this study caused root hair curling (Fig. 4.1) and formed small callus-like structures that were unable to fix nitrogen on the roots of *L. leucocephala* (Fig. 3.3, Table 4.1). Sections through the calli showed a disorganized group of small plant cells often containing osmiophilic droplets (OPD) which are not observed in normal nitrogen-fixing

nodules (Fig. 4.2). Some of the ranges of callus structures found are illustrated in Figs. 4.3-4.5. In some callus-like structures there was little obvious meristematic zone or the presence of infection threads or vascular bundles and certainly no bacteroid zone, although there were extensive areas of plant cells containing osmiophilic droplets (Fig. 4.3, 4.5 a, b). Other calli had small meristematic zones with infection threads, less cells containing OPD, limited bacterial release from infection threads, and some enlarged plant cells. Vascular bundles were also present (Fig. 4.4). Furthermore, large numbers of osmiophilic droplets were found in the lateral root cells connected to the small callus (Fig. 4.5 c). Thus, these Muc^- mutants with a marked alteration of their surface polysaccharides were defective in establishing even a nodule. No reversion of the mutant phenotype was observed with any of the Muc^- mutants used.

4.3 NODULATION OF *LEUCAENA* BY $MUC^-NOD^+FIX^-$ (CALLUS-FORMING) MUTANTS MIXED WITH A MUC^+NOD^- MUTANT

Strain ANU265 (a Sym plasmid-cured derivative of strain NGR234, Nod^- on all host plants) was used as the Muc^+Nod^- strain and mixed with various $Muc^-Nod^+Fix^-$ (callus-forming) mutants and inoculated onto *Leucaena* plants. Results presented in Table 4.1 show: a) that 15 out of 17 combinations induced normal-sized nodules on *Leucaena* plants instead of calli and b) that in 11 cases a proportion of the nodules were able to reduce acetylene. The fraction of test plants giving a Nod^+Fix^+ phenotype ranged from 25-87%. Acetylene reduction assays on four-week old plants showed between 16-39% of the activity of the parent strain ANU280 (Table 4.1).

Microscopic examination of nodule tissue showed that the Fix^+ nodules produced by mixed inocula resembled the nodules produced by the parent strain ANU280 (Fig. 4.2), as they contained no osmiophilic droplets and had vascular bundles and an extensive bacteroid zone (Fig. 4.5 e, f).

Both Muc^- and Muc^+ bacteria were isolated from crushed nodules. In some nodules, up to 80% of the re-isolated bacteria were the Muc^+Nod^- strain ANU265 (Table 4.2). The genetic markers and symbiotic properties of these re-isolates were identical to the original inoculum strains indicating that there was no genetic exchange between Muc^- and the Muc^+ mutants. In some combinations of Muc^- mutants and strain ANU265, the nodules formed were small and ineffective (Table 4.1). They still had a normal nodule structure with vascular bundles, but it appeared the plant cells were not infected by bacteria (Fig. 4.5 d). These Fix^- nodules still had a considerable number of osmiophilic droplets present (Fig. 4.5 d). Several combinations of mutant bacteria used to inoculate *Leucaena* plants failed to "cooperate" and induce nodules (Table 4.1). Out of all the combinations, the most successful pair was the mixture of strains ANU2840 and ANU265 which effectively nodulated 87% of *Leucaena* plants. The acetylene reduction activity of the nodules formed was 39% of the parent strain ANU280 (Table 4.1).

4.4 COINOCULATION OF MUC^- MUTANTS WITH A MUC^- ANU265 DERIVATIVE

To determine if the EPS produced by strain ANU265 was essential for effective nodulation of *Leucaena* in coinoculation experiments, a Muc^- derivative of strain ANU265 was isolated. A Muc^-Nod^- derivative

of strain ANU265 was constructed using a kanamycin-sensitive derivative of plasmid R68.45 [which has chromosomal mobilization ability (Cma^+)] to transfer the Tn*5* mutation of Muc^- mutant ANU2811 into the genome of strain ANU265. Strain ANU2811(R68.45) was used as the donor in the mating with strain ANU265 and selection was made for the transfer of the kanamycin resistant marker of transposon Tn*5*. Kanamycin-resistant transconjugants arose at a frequency of 10^{-9}. The colony morphology of recombinants was similar to that of strain ANU2811 showing that the Muc^- phenotype was transferred with the Tn*5* mutation. This was verified by the following findings: a) all Km^r isolates contained a megaplasmid and plasmid R68.45 but did not carry the Sym plasmid; b) the Km^r isolates had a copy of Tn*5* which was located in the same sized DNA fragment as mutant ANU2811 (Fig. 4.6); and c) all Km^r isolates retained a Nod^- phenotype (Table 4.3). This Muc^-Nod^- ANU265 derivative was named ANU266. The level of EPS produced by strain ANU266 was similar to that produced by strain ANU2811 (Table 4.3). When strain ANU266 was mixed with strain ANU2840, the combination was unable to form an effective nodule on *Leucaena* plants and only callus-like structures were produced (Table 4.3). These callus-like structures were identical in appearance to those induced by strain ANU2840 alone. Similarly, combination of two different $Muc^-Nod^+Fix^-$ (callus-forming) mutants, for example, ANU2840 and ANU2811, failed to produce nitrogen-fixing nodules (Table 4.3). These results indicated that it was the EPS produced by Muc^+Nod^- strain ANU265 that was essential for the effective nodulation of *Leucaena* plants.

4.5 NODULATION OF *LEUCAENA* BY MIXED INOCULA THAT PRODUCE VARIABLE AMOUNTS OF EPS

Individual R-prime plasmids carrying *Rhizobium* DNA flanking the *Tn5* insertion sites of several Group 2 Muc^- mutants have been isolated using the plasmid R68.45 Km^S (Chapter eight). The transfer to the parent strain ANU280 of one of these R-prime plasmids, which contained *Rhizobium* DNA flanking the ANU2840 mutation (R'2840), resulted in ANU280 transconjugants possessing a non-mucoid phenotype (Muc^-). Similarly, transfer of this R-prime to strain ANU265 also resulted in transconjugants with a Muc^- phenotype. Furthermore, the Muc^- colony morphology did not persist if the transconjugants were incubated for prolonged periods on selective BMM plates. The initial phenotype of the transconjugants (i.e. Muc^-) was similar to that of mutant ANU2840. However, with prolonged incubation (longer than 5 days) strain ANU265(R'2840) gradually formed slightly mucoid colonies (Fig. 4.7).

To determine if the EPS produced by strain ANU265(R'2840) following extended growth on BMM medium had any effect on the result of the coinoculation experiment, a further study was undertaken. Strain ANU265(R'2840) was incubated for different times to allow the bacteria to produce varying amounts of EPS. These transconjugant colonies were then mixed with strain ANU2840 and inoculated onto *Leucaena* plants. The amount of EPS present in the ANU265(R'2840) preparation used for the coinoculation was analyzed by hexose determinations (see Materials and Methods). The coinoculation of the strain ANU2840 with 3 day cultured ANU265(R'2840) produced nodules (rather than calli), but these were lacking in nitrogen-fixing ability (Table 4.4). By increasing the culture time of strain ANU265(R'2840)

on BMM agar plates prior to coinoculation experiments, a gradual restoration of the nitrogen-fixing ability resulted in the nodules formed (Table 4.4). This increase was concomitant with an enlargement of the nodules produced by the coinoculation (Table 4.4) and is directly correlated to the amount of EPS which the strain ANU265(R'2840) had produced prior to the coinoculation assay (Fig. 4.8).

4.6 EFFECT OF DEAD BACTERIA ON COOPERATIVITY BETWEEN DIFFERENT STRAINS

If strain ANU265 was heat-killed before being mixed with Muc^- mutant ANU2840 and inoculated onto *Leucaena* plants, small and ineffective nodules were formed (Table 4.3). The bacteria re-isolated from these nodules were Muc^- and had the same genetic markers and symbiotic properties as strain ANU2840. If Muc^- strain ANU2840 was heat-killed and mixed with strain ANU265, no nodules or calli were formed on *Leucaena* plants (Table 4.3).

4.7 DISCUSSION

Exopolysaccharide-deficient mutants of the fast-growing broad host range *Rhizobium* strain NGR234 caused root hair curling (Hac^+ phenotype) but did not nodulate the tropical legume *Leucaena leucocephala*. Instead, these Muc^- mutants induced callus-like structures on the lateral roots. The calli formed by the various Muc^- mutants varied in size. Studies using light microscopy showed that these calli could range from having no obvious meristematic zone,

vascular bundles and infection threads to those having a small meristematic zone, vascular bundles and infection threads. No bacteroid-packed plant cells could be observed in these calli (Figs. 4.3-4.5). Thus, these callus-like structures are most probably deformed nodules which result from an early inhibition of infection of the Muc^- mutants on *Leucaena* plants. An important feature of these calli was the presence of osmiophilic droplets (Figs. 4.3-4.5). Osmiophilic droplets are often observed in plant hypersensitive reactions induced by many avirulent pathogens (Mueller and Beckman, 1974; Roebuck *et. al.*, 1978). These droplets may represent localized accumulation of phenolic compounds, such as phytoalexins (antibacterial compounds of low molecular weight) which inhibit the growth of invading organisms (Gnanamanickam and Patil, 1977). Phytoalexins are present in many legume plants but, in healthy plant tissues, the concentration of phytoalexins is very low or undetectable (Stoess, 1980). When plants are inoculated with avirulent pathogens, however, phytoalexins can accumulate rapidly. Once production is initiated at the infection site, phytoalexin accumulation may be extremely high, reaching 10% or more of the tissue dry weight (West, 1981). Such levels greatly exceed those at which most plant pathogens are sensitive. Thus, it can be concluded that the Muc^- mutants are probably inducing an avirulent response on *Leucaena* plants. *Leucaena* plants may recognize these avirulent strains at an early infection stage and produce antibacterial compounds, which inhibit further bacterial invasion. In addition, osmiophilic droplets occur in the lateral roots connected to the small calli, suggesting that the plant response induced by Muc^- mutants is effective at least over a small distance.

The present results demonstrate that the inability of EPS-deficient mutants to form normal nitrogen-fixing nodules on *Leucaena* plants can be functionally complemented by a $Muc^{+}Nod^{-}$ strain. The Muc^{+} strain with no Sym plasmid can enter the nodule alone with the invasive Muc^{-} strain and cause induction of nitrogen-fixing nodules ("helper activity"). This phenomenon is similar to that found in clover plants inoculated with mixtures of $Muc^{-}Nod^{+}Fix^{-}$ and $Muc^{+}Nod^{-}$ mutants of *Rhizobium trifolii* (Rolfe *et. al.*, 1980 b). Chemical studies of surface polysaccharides with *R. trifolii and R. japonicum* strains have shown that the Sym-plasmid-cured strains do not have impaired synthesis of EPS but do have altered synthesis of lipopolysaccharides (LPS)(Carlson *et. al.*, 1986; Carlson and Yaday, 1985). Results presented in this study are consistent with these findings, as the structure of the EPS produced by strain ANU265 is identical to that induced by ANU280. This has been confirmed by recent data (Djordjevic *et. al.*, 1986). Thus, it is likely that the EPS produced by the $Muc^{+}Nod^{-}$ strain is essential for the "helper effect" with Muc^{-} strains in the establishment of a normal Fix^{+} nodule on *Leucaena* plants. This possibility was further supported by the findings: a) that the constructed Muc^{-} mutant of strain ANU265 was unable to enhance the nodulation ability of $Muc^{-}Nod^{+}Fix^{-}$ (callus-forming) strain ANU2840 on *Leucaena* plants, and b) that the effective nodulation of *Leucaena* plants by the mixed inocula of strains ANU2840 and ANU265(R'2840) was correlated with an increase of EPS production by the transconjugant strain. Furthermore, this strain interaction was more pronounced when viable cells were used, although the heat-killed strain ANU265 did lead to small nodules, rather than calli. This suggests that the EPS is important for proper nodule development

after the Muc$^-$ strains invade the root cells. It is also possible that the heat-killed bacteria release something that elicits plant defence response at later infection stages.

The interactions between four of the Muc$^-$ strains and strain ANU265 led to normal-sized nodules on *Leucaena* plants but these were non-nitrogen-fixing. However, two of the Muc$^-$ strains were unable to cooperate with strain ANU265 to form nodules on *Leucaena* plants. Clearly, this is a complex interaction and thus requires further analysis of the various mutant strains. Unsuccessful interactions by different Muc$^-$ mutants may be due to a variety of alterations in their surface components which elicit a strong plant reaction. The present results suggest that the *Rhizobium* EPS is important at an early stage of the infection process. It may have a passive role as a protective barrier for the invading bacterial cell or perhaps a more potentially controversial role as a regulator of plant gene expression.

4.8 ACKNOWLEDGEMENTS

Most of the results shown in this chapter have appeared in the papers "Cooperativity between *Rhizobium* mutant strains: Induction of nitrogen-fixing nodules on the tropical legume *Leucaena leucocephala*" by Hancai Chen, and Barry G. Rolfe in Journal of Plant Physiology, volume 127: 307-322 (1987) and "Alteration of the effective nodulation properties of fast-growing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis" by Hancai Chen, Michael Batley, John Redmond and Barry G. Rolfe in Journal of Plant Physiology, volume 120: 331-349 (1985).

Table 4.1 Effect of strain ANU265(Muc^+Nod^-) on the nodulation of *Leucaena* by Muc^- mutants

Strains	Mucoid group	No. of plants tested	No. of plants forming			
			calli	Fix^- nodules	Fix^+ nodules	C_2H_2# reduction
ANU280	Wild-type	17	0	0	17	19
ANU265		20	0	0	0	0
ANU2885	1	12	12	0	0	< 0.1
ANU2811	2	12	12	0	0	< 0.1
ANU2820	2	10	10	0	0	< 0.1
ANU2831	2	8	8	0	0	< 0.1
ANU2838	2	8	8	0	0	< 0.1
ANU2840	2	23	23	0	0	< 0.1
ANU2841	2	8	8	0	0	< 0.1
ANU2852	2	8	8	0	0	< 0.1
ANU2854	2	8	8	0	0	< 0.1
ANU2867	2	8	8	0	0	< 0.1
ANU2808	2	8	8	0	0	< 0.1
ANU2822	2	7	7	0	0	< 0.1
ANU2844	2	8	8	0	0	< 0.1
ANU2890	2	8	8	0	0	< 0.1
ANU2875	2	6	6	0	0	< 0.1
ANU2801	4	8	8	0	0	< 0.1
ANU2814	5	8	8	0	0	< 0.1
ANU2885+ANU265		12	7	2	3	2.3
ANU2811+ANU265		12	4	0	8	3.2
ANU2820+ANU265		10	2	2	6	6.7
ANU2831+ANU265		8	2	4	2	4.8
ANU2838+ANU265		8	2	4	2	3.0
ANU2840+ANU265		23	2	1	20	7.3
ANU2841+ANU265		8	1	4	3	3.0
ANU2852+ANU265		8	0	6	2	3.6
ANU2854+ANU265		8	2	2	4	3.5
ANU2867+ANU265		8	2	2	4	6.3
ANU2808+ANU265		8	3	5	0	< 0.1
ANU2822+ANU265		7	2	5	0	< 0.1
ANU2844+ANU265		8	4	4	0	< 0.1
ANU2890+ANU265		8	4	4	0	< 0.1
ANU2875+ANU265		6	6	0	0	< 0.1
ANU2801+ANU265		8	8	0	0	< 0.1
ANU2814+ANU265		8	2	3	3	3.6

The activity of acetylene reduction was expressed as: n moles C_2H_4/mg fresh nodule/hr. Each data point was an average acetylene reduction activity of Fix^+ plants.

Table 4.2 Strain content of nodules formed by mixed inocula

Strains in cell mixtures	No. of nodules tested	Percentage range of each strain in nodule crushes
ANU2808 and ANU265	8	30-80 (ANU2808)
		20-70 (ANU265)
ANU2811 and ANU265	8	20-70 (ANU2811)
		30-80 (ANU265)
ANU2814 and ANU265	6	50 (ANU2814)
		50 (ANU265)
ANU2820 and ANU265	7	30-70 (ANU2820)
		30-70 (ANU265)
ANU2822 and ANU265	6	20-70 (ANU2822)
		30-80 (ANU265)
ANU2831 and ANU265	7	20-50 (ANU2831)
		50-80 (ANU265)
ANU2838 and ANU265	8	50-60 (ANU2838)
		40-50 (ANU265)
ANU2840 and ANU265	10	40-60 (ANU2840)
		40-60 (ANU265)
ANU2852 and ANU265	8	30-80 (ANU2852)
		20-70 (ANU265)
ANU2854 and ANU265	6	50-60 (ANU2854)
		40-50 (ANU265)
ANU2867 and ANU265	8	70-90 (ANU2867)
		10-30 (ANU265)
ANU2890 and ANU265	8	40-60 (ANU2890)
		40-60 (ANU265)

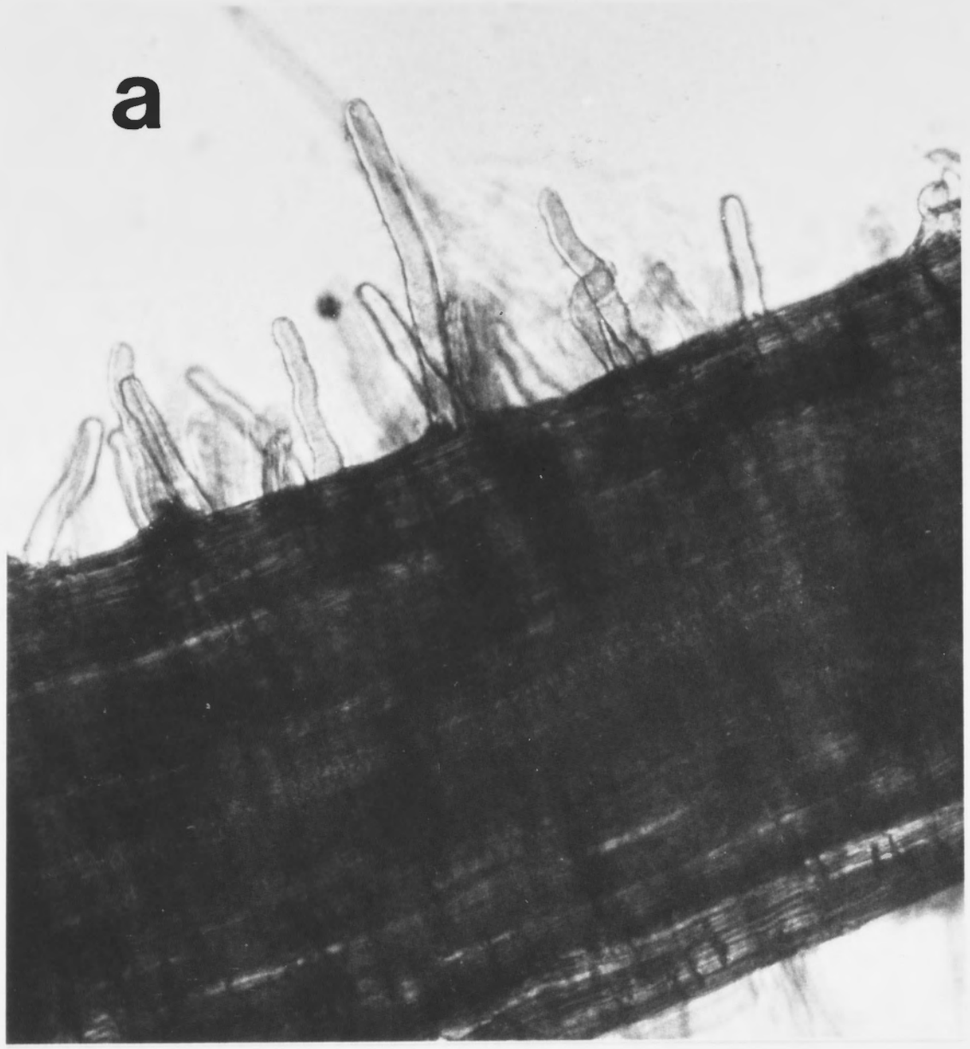
Table 4.3 Effect of EPS production and strain viability on strain cooperativity for the nodulation of *Leucaena*

Inoculating strains	EPS production (µg hexose/ mg protein)	Plant response	C_2H_2 reduction (n moles C_2H_4/ mg fresh nodule/hr)
ANU280	465	nodules	22
ANU2840	35	calli	< 0.1
ANU265	471	no nodules	
ANU2840 and ANU265		nodules	7.7
ANU266	39	no nodules	
ANU2840 and ANU266		calli	< 0.1
ANU2811	33	calli	< 0.1
ANU2840 and ANU2811		calli	< 0.1
ANU265 heat killed		no nodules	
ANU2840 and ANU265 heat killed		small nodules	< 0.1
ANU2840 heat killed		no nodules	
ANU2840 heat killed and ANU265		nodules	

Table 4.4. Effect of strain ANU265(R'2840) EPS on the nodulation of *Leucaena* by the strain ANU2840. Strain ANU265(R'2840) was grown for various times on BMM plates, mixed in equal proportions with strain ANU2840 (cultured for 2 days on BMM plates), and then this mixed inoculum was used to infect *Leucaena* seedlings. The transconjugant ANU265(R'2840) was constructed by mating *E. coli* strain HB101(R'2840) with *Rhizobium* strain ANU265 overnight on a TY plate. The mating cultures were diluted to 100 ml with sterile water and 0.2 ml was spread on each of 50 large patch dishes containing BMM agar medium supplemented with 200 µg/ml of kanamycin and 100 µg/ml of spectinomycin. Colonies appeared after 2 days incubation at 29°C. In each mixing experiment, about 3000 colonies were scraped from 5 plates and used for the preparation of the mixed inoculum with strain ANU2840. At the time the mixed inoculum was prepared, strain ANU265(R'2840) was assayed to determine the amount of EPS which had been produced. Plants were scored for nodule number and tested for acetylene reduction after four weeks.

Transconjugant ANU265(R'2840)		Strain ANU2840 mixed with transconjugant ANU265(R'2840)					
Incubation time (Days)	EPS produced (µg hexose/ mg protein)	Number of plants tested	Total number of nodules formed	Fesh weight of total nodules	Fresh weight of average nodule	n moles C_2H_4/hr (total nodules activities)	n moles C_2H_4/mg fresh nodule/hr
3	88	10	46	18	0.39	0	0
5	110	10	32	13	0.41	9	0.7
6	150	10	28	15	0.54	27	1.8
7	190	10	32	33	1.03	76	2.3
8	270	10	30	43	1.43	240	5.6

Fig. 4.1 The response of *Leucaena leucocephala* root hair cells on lateral roots exposed to the Muc$^-$ mutant ANU2840. a) Root hairs without inoculation; b) root hair curling induced by mutant ANU2840.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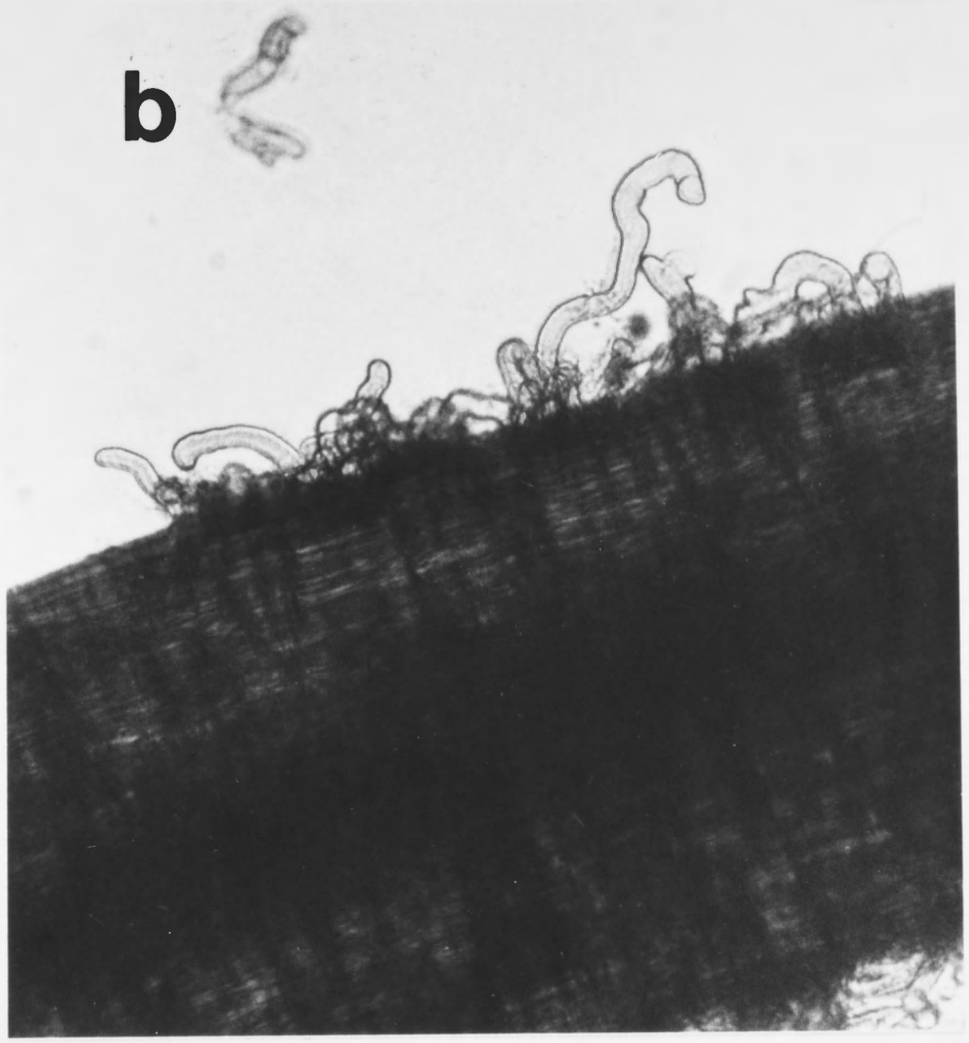
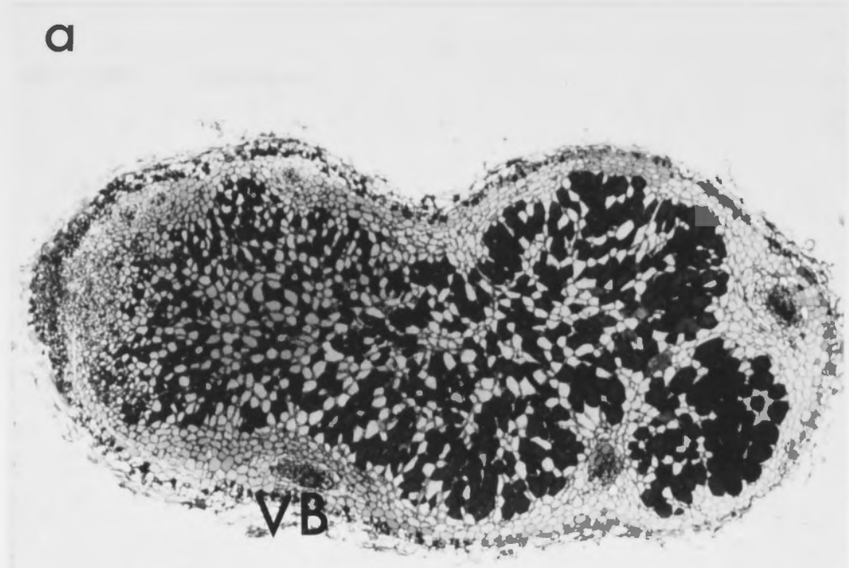
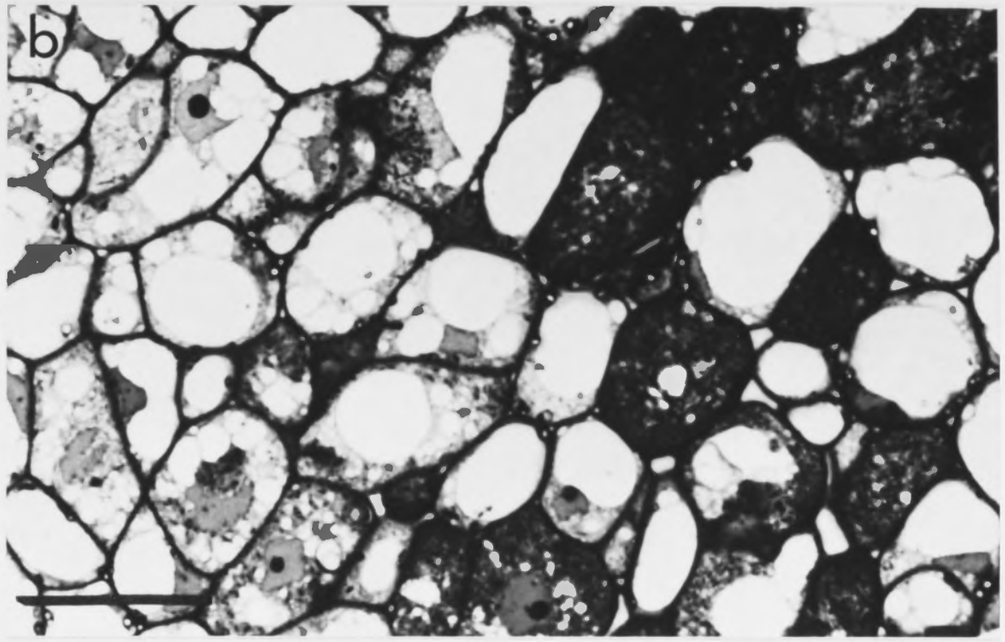
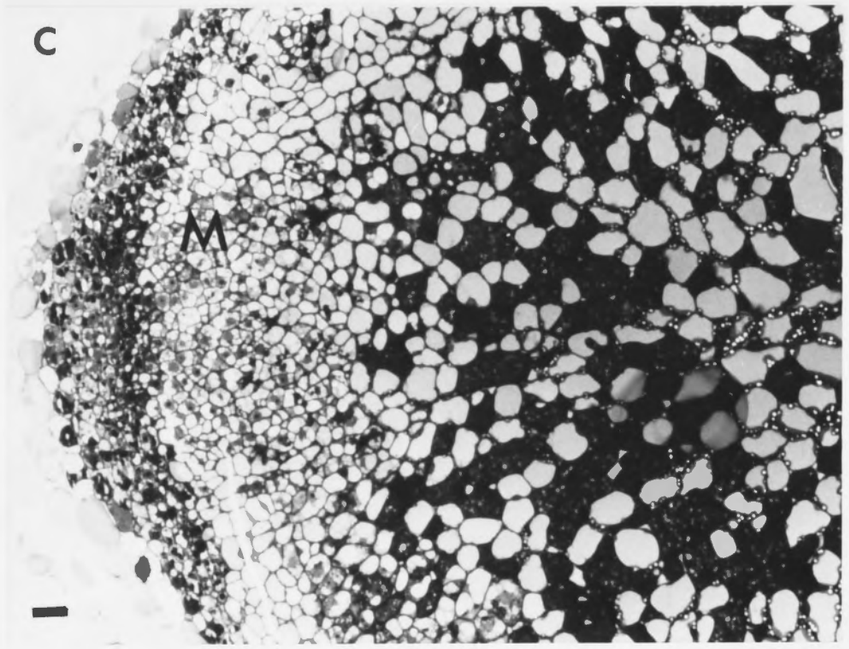

b

Fig. 4.2 Structure of nitrogen-fixing nodules formed on *Leucaena* by parent strain ANU280. a) Longitudinal section showing different zones of infected tissue and the vascular bundles (VB); b), c) meristematic (M) and early symbiotic and infection thread invasion zones; d) later symbiotic zone with plant cells packed with bacteroids and some uninfected cells. Bar represents 0.5 mm in a) and 100 µm for b), c) and d).


a
VB


b


c
M

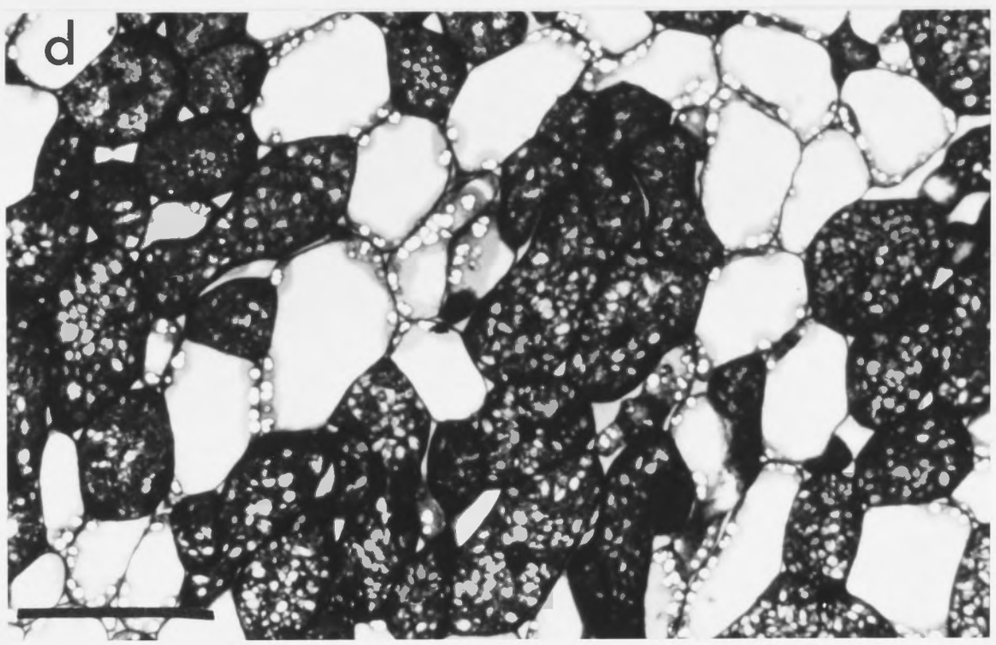
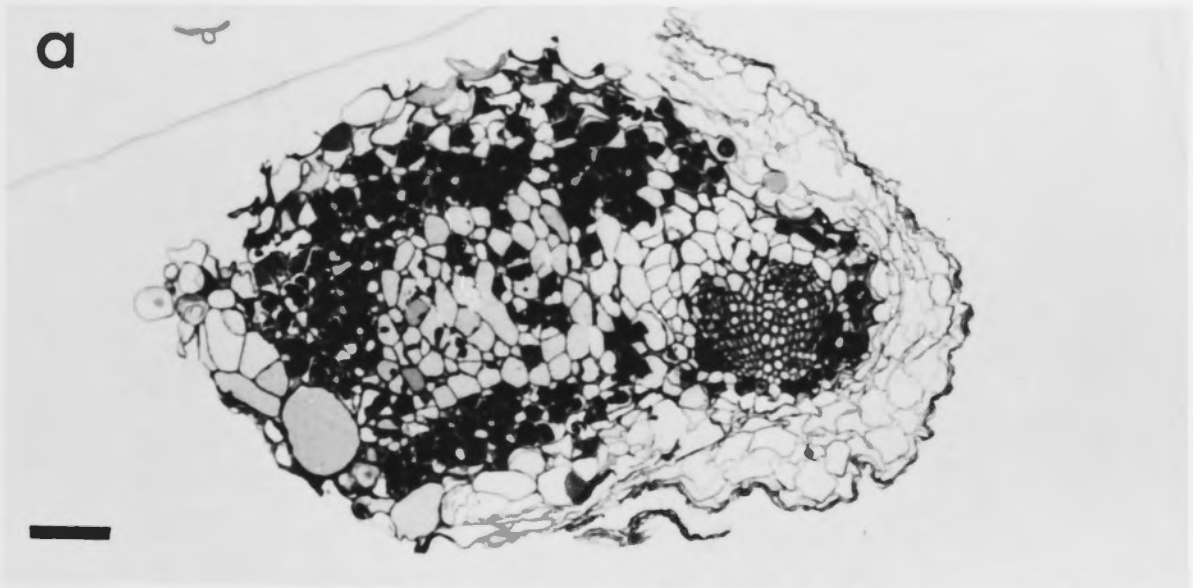
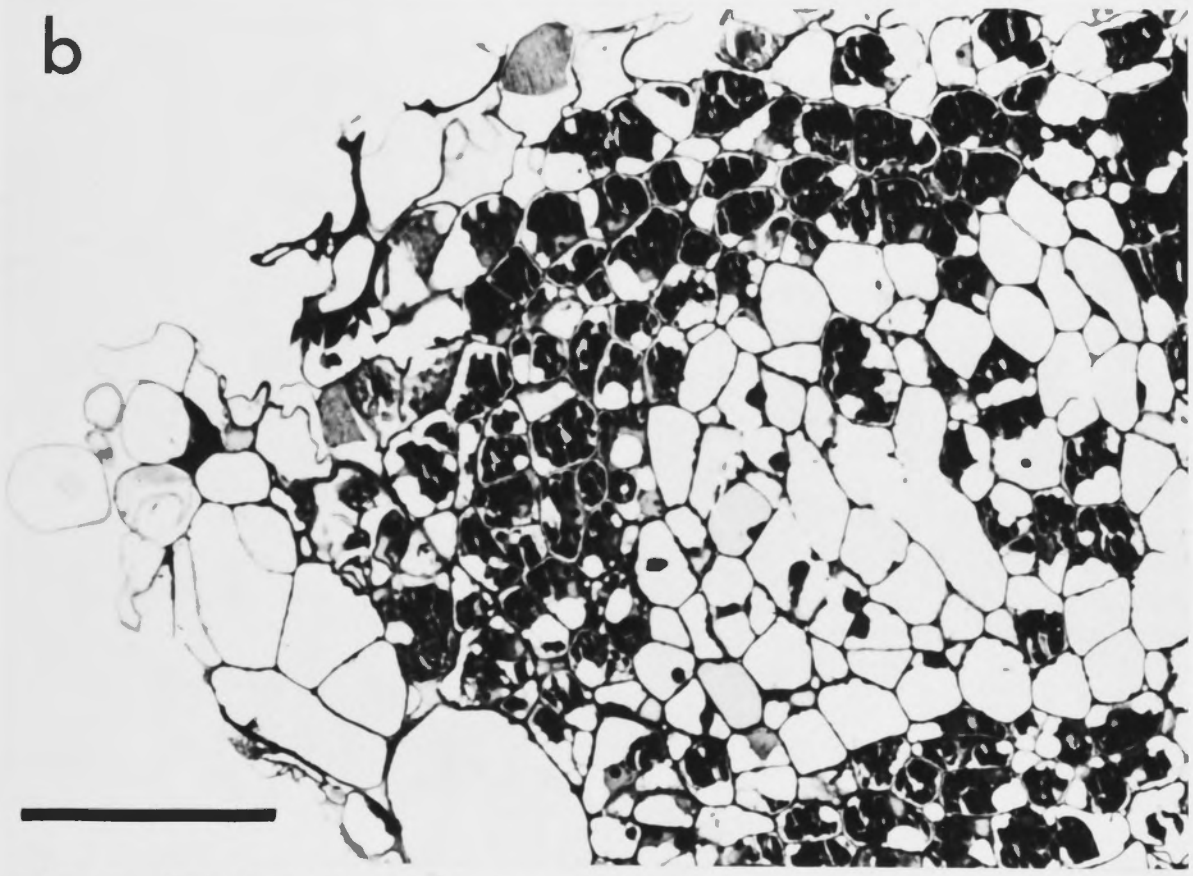

d

Fig. 4.3 Small callus-tumour-like structures formed on *Leucaena* roots. a) Mutant ANU2825 (Group 4) like most Group 2 mutants induces small callus-like structures which have small plant cells of the size of those found in the meristematic zone of nodules induced by the parent strain (Fig. 4.2a); b) and C) there were no obvious vascular bundles or large plant cells comparable to those observed in nodules induced by strain ANU280 (Fig. 4.2b-d). In addition, the majority of the plant cells contained osmiophilic droplets. Bar represents 100µm.


a


b

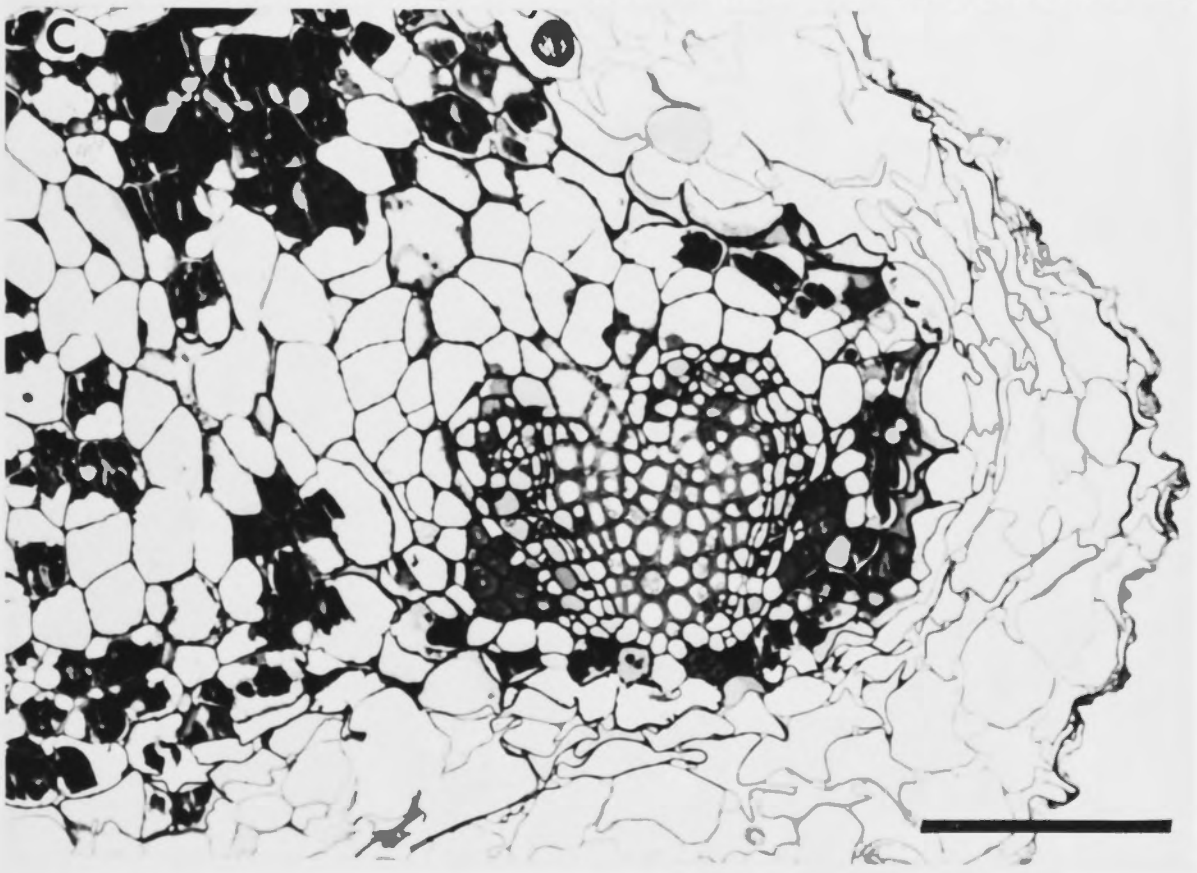
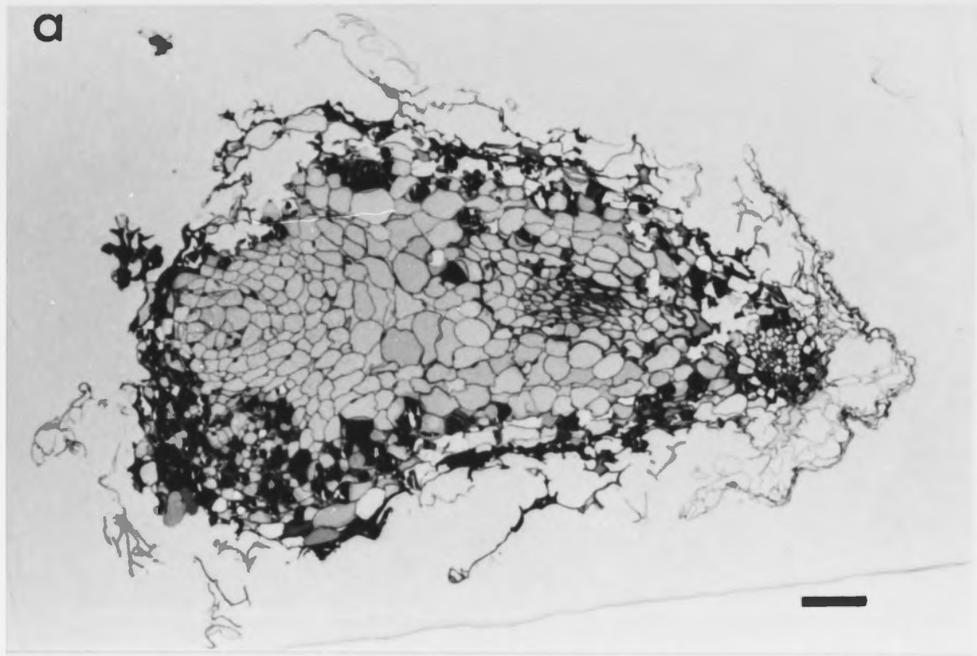
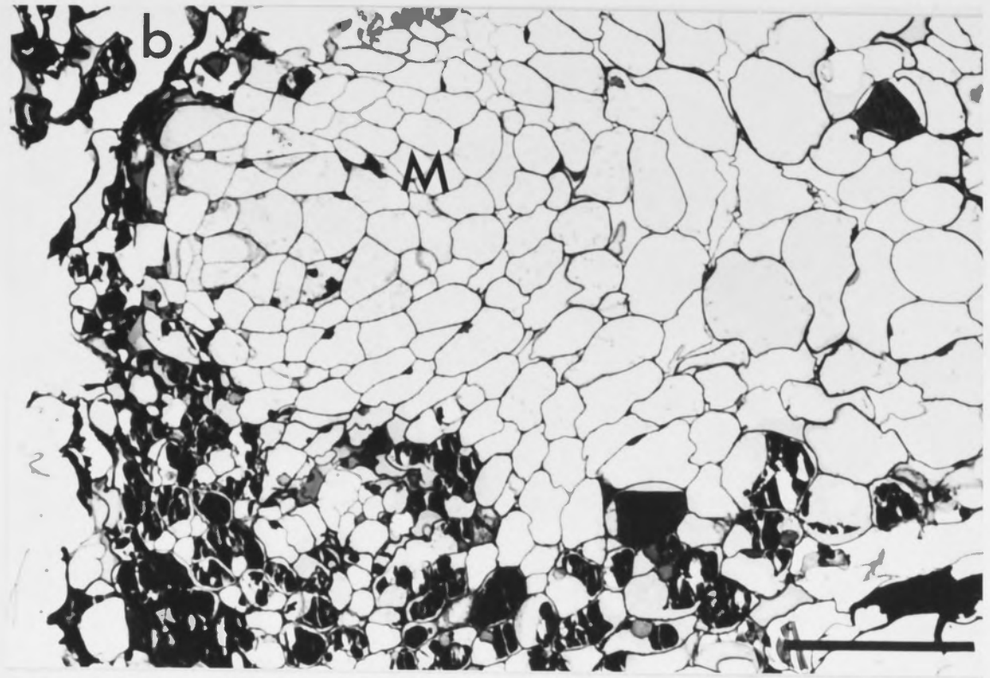
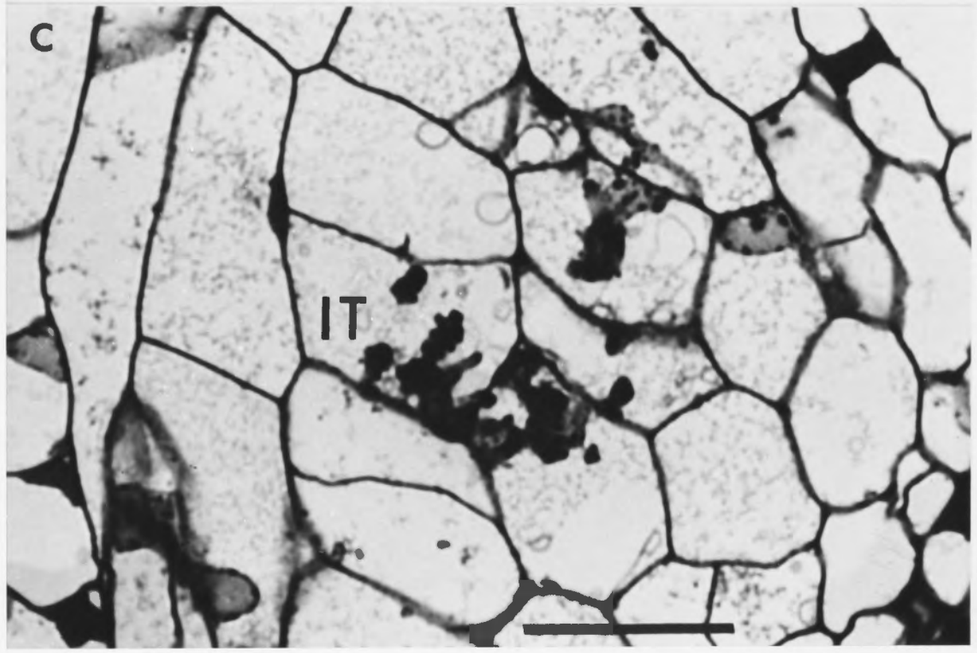

c
d

Fig. 4.4 Large callus-tumour-like structures formed on *Leucaena* roots. a) Mutant ANU2814 (Group 5) induces a larger disorganized outgrowth on roots; b) a definite meristematic (M) zone is formed; c) some infection threads (IT) can be observed in the meristematic zone; d) large empty plant cells and some vascular bundles (VB) were also observed. Bar represents 100 µ in a), b) and d) and 50 µm in c).


a


b
M


c
IT

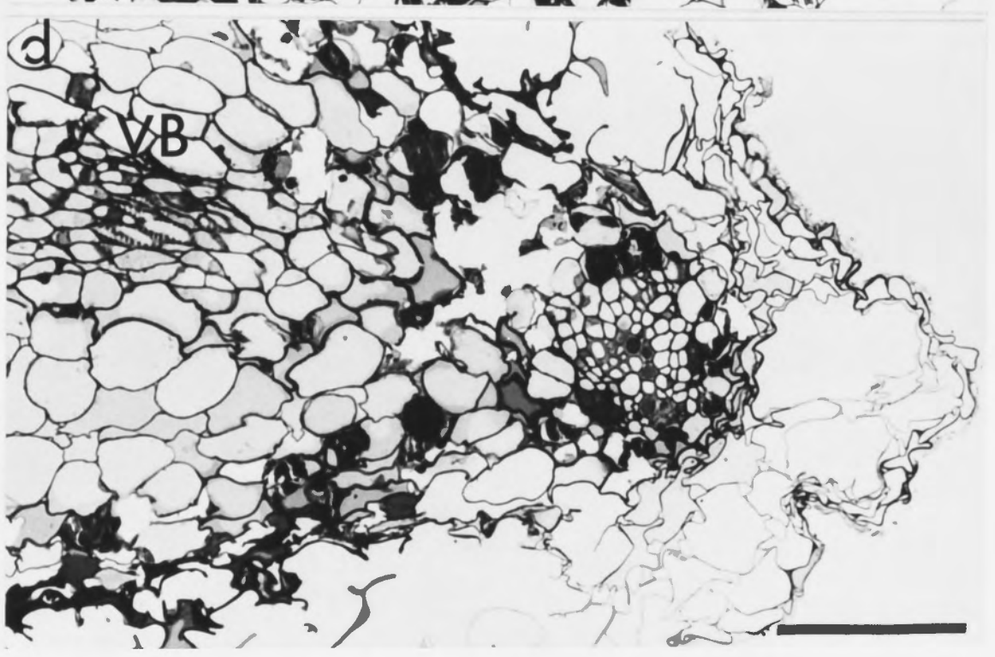

d
VB

Fig. 4.5 Light Microscopic examination of *L. leucocephala* callus-like structures and nodules. a) Small callus-like structures induced by Muc$^-$ mutant ANU2840 showing an abnormal structure, no obvious meristematic activity, nor vascular bundles or large plant cells packed with bacteroids comparable to those observed in nodules induced by the parent strain ANU280 (Fig. 4.2) were present; b) close-up view of the nodule formed by mutant ANU2840 showing small empty plant cells and the plant cells containing osmiophilic droplets; c) lateral root connected to the small callus formed by ANU2840 showing many osmiophilic droplets within the lateral root; d) non-nitrogen-fixing nodule induced by a mixture of strain ANU2808 and strain ANU265 showing vascular bundles (VB) and emptied plant cells. However, many of the plant cells also contain osmiophilic droplets; e) nitrogen-fixing nodule infected with strain ANU2840 and strain ANU265 showing vascular bundles and many plant cells filled with bacteroids; f) close-up view of the nodule formed by the mixture of strains ANU2840 and ANU265 showing bacteroid development and vascular bundle. Bar represents 100 µm in a), c), d) e) and 50 µm in b), f).

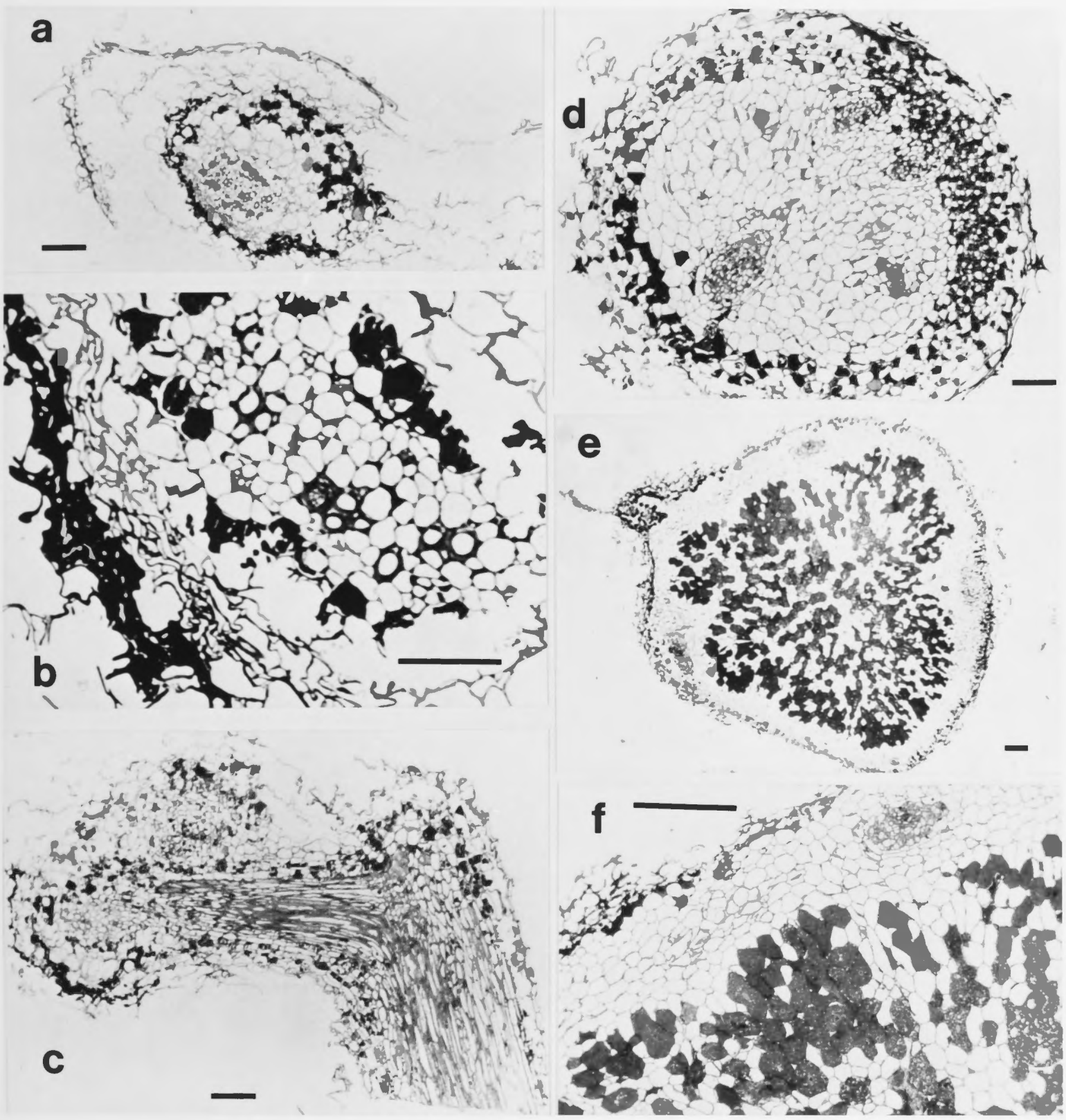

a
b
c
d
e
f

Fig. 4.6 Mobilization of the transposon Tn*5* from mutant ANU2811 into strain ANU265 with the kanamycin-sensitive derivative of plasmid R68.45. (A) Total DNA digested with *Eco*RI endonuclease and electrophoresed in a 1% agarose gel. Lane 1, phage λ DNA digested with *Hin*dIII; lane 2, ANU265; lane 3, ANU266; Lane 4, ANU2811; (B) the digested DNA fragments in the agarose gel transferred to nitrocellulose sheet and hybridized to ^{32}P-labelled Tn*5*-containing plasmid, pkan-2.

^{32}P-labelled lambda markers are shown in Lane 1.

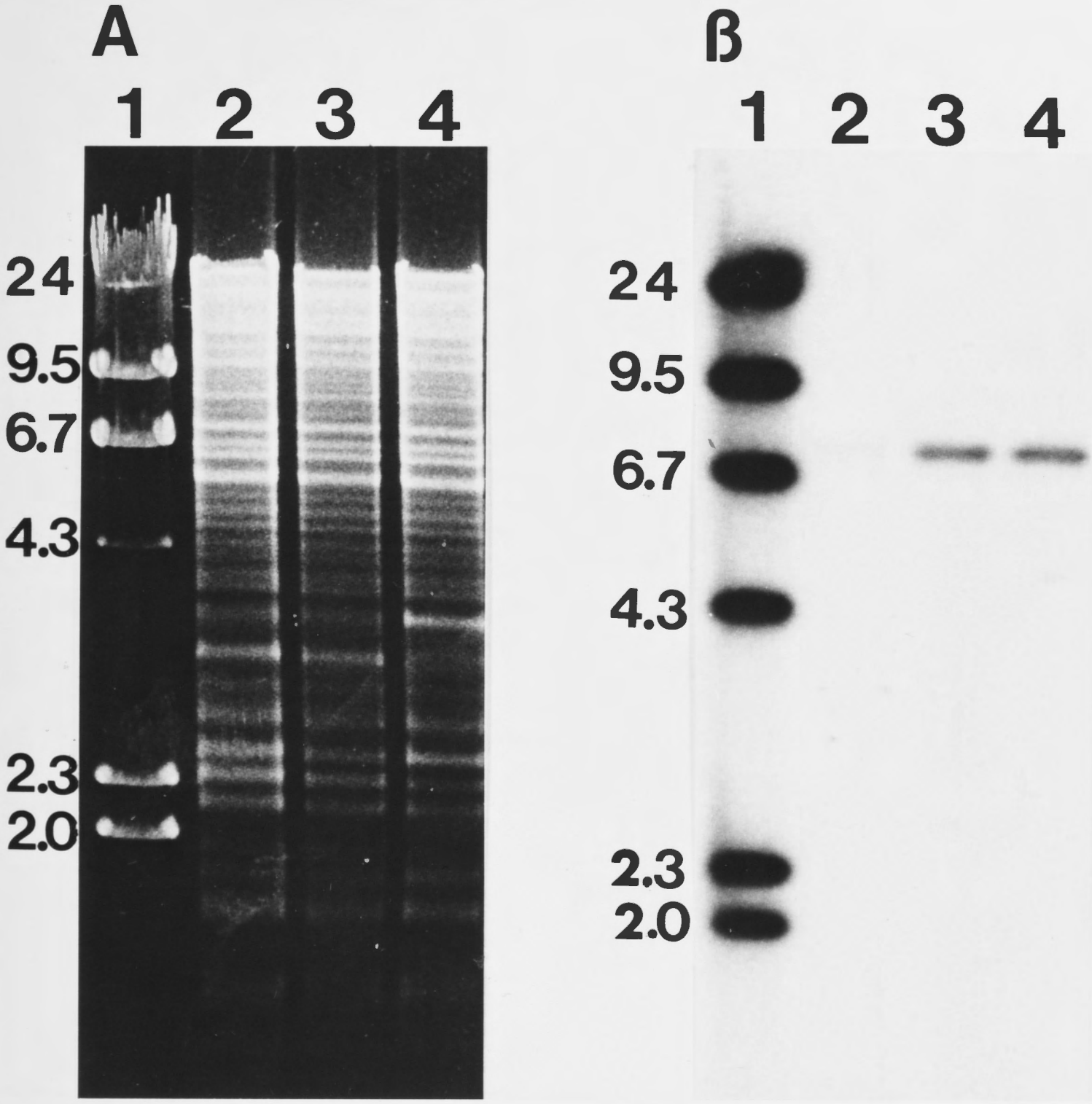

A
1 2 3 4
24
9.5
6.7
4.3
2.3
2.0
ß
1 2 3 4
24
9.5
6.7
4.3
2.3
2.0

Fig. 4.7 Comparison of the colony morphologies of strain ANU265 and its derivatives. All strains were grown on BMM agar medium with a supplement of 100 µg/ml of spectinomycin for strain ANU265 or 200 µg/ml of kanamycin for strain ANU2840 or 100 µg/ml of spectinomycin and 200 µg/ml of kanamycin for strain ANU265(R'2840). a) ANU2840, a rough colony morphology; b) ANU265, mucoid colonies; c) ANU265(R'2840) after 3 days incubation, a rough phenotype; d) ANU265(R'2840) after 5 days incubation, a semi-rough colony morphology.

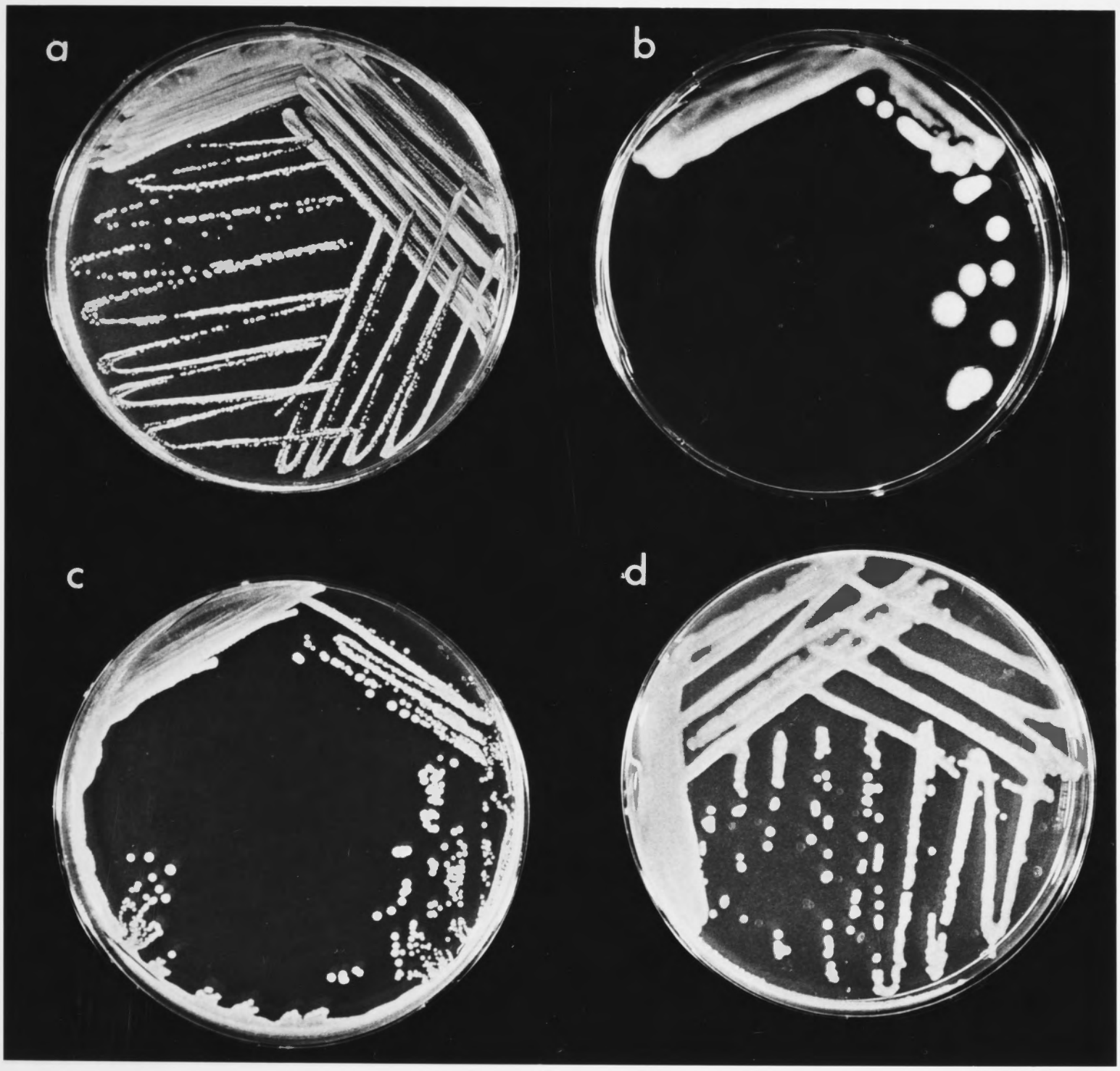
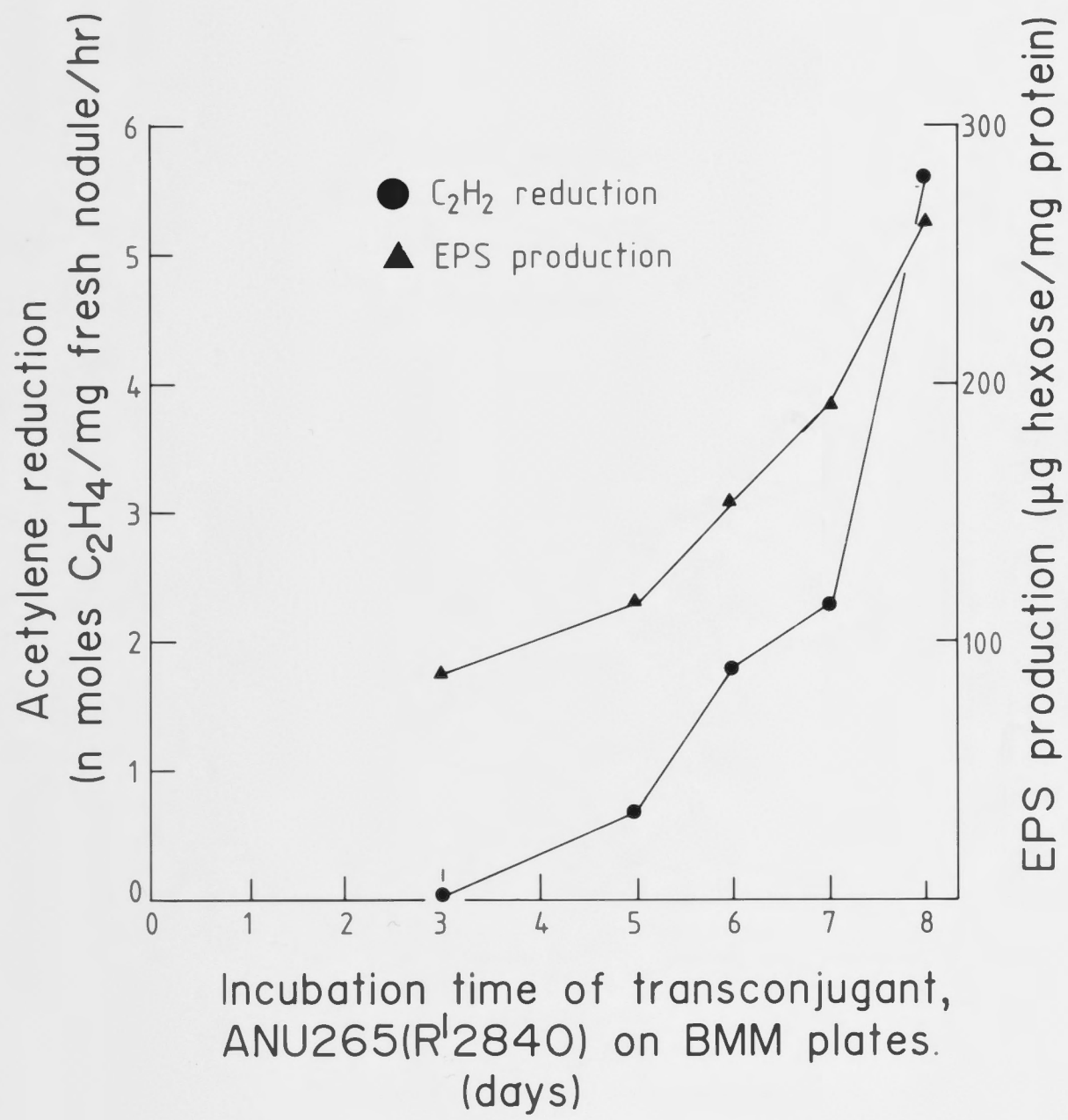

a
b
c
d

Fig. 4.8 Correlation between EPS production of the strain ANU265(R'2840) and the effective nodulation of *Leucaena* following coinoculation with strains ANU2840 and ANU265(R'2840). The data are from Table 4.4.


Acetylene reduction
(n moles C_2H_4/mg fresh nodule/hr)
EPS production (µg hexose/mg protein)
C_2H_2 reduction
EPS production
0
1
2
3
4
5
6
100
200
300
0
1
2
3
4
5
6
7
8
Incubation time of transconjugant,
ANU265(R'2840) on BMM plates.
(days)

CHAPTER FIVE

EFFECTIVE NODULATION OF *PARASPONIA* BY MUTANT ANU2895

5.1 INTRODUCTION

The nodulation of the nonlegume plant *Parasponia* by *Rhizobium* strains occurs through a mechanism quite distinct from that used by the same strains nodulating a legume species (Trinick and Gallbraith, 1976; Lancelle and Torrey, 1984; Price *et. al.*, 1985). In contrast to the invasion via root hair curling, infection thread formation and bacterial release observed during legume nodulation (Ridge and Rolfe, 1986), bacteria invade *Parasponia* roots through points of bacterium-induced meristematic activity which break the epidermal surface of the root allowing bacteria direct access to cortical cells (Bender *et. al.*, 1987b). These cells become invaded via infection thread formation and nitrogen fixation occurs within these threads (Trinick, 1979). The fully developed *Parasponia* nodule resembles a modified lateral root structure with a central vascular system (Trinick, 1979). As with legume nodulation, there is also a host-specific component to non-legume nodulation. Only certain strains of *Rhizobium* and *Bradyrhizobium* are capable of nodulating *Parasponia* and these strains do so with varying efficiency (Trinick and Galbraith, 1980).

The fast-growing broad host-range *Rhizobium* strain NGR234 is able to form nitrogen-fixing nodules on a large number of tropical legumes (Trinick, 1980 b). This strain also nodulates *Parasponia andersonii* but the nodules formed are unable to fix nitrogen (Trinick and Galbraith, 1980). Chapter 3 showed that Tn*5*-induced mutants of strain

NGR234 with altered exopolysaccharide production had various phenotypes on five different tropical legumes. In this chapter, some of these mutants were chosen as inoculum to infect *Parasponia andersonii* and ask the question about whether these surface alterations could affect the symbiotic properties of strain NGR234 on the non-legume plant.

5.2 PLANT RESPONSE TO EXOPOLYSACCHARIDE-SYNTHESIS MUTANTS

Seventeen mucoid mutants of strain ANU280 (streptomycin- and rifampicin-resistant derivative of strain NGR234) from seven different groups (Table 3.1) were used as inoculum to infect *Parasponia andersonii*, using the plate assay method (Bender and Rolfe 1985). All of these mutants are induced by a single Tn*5* transposon insertion probably located on chromosome (Table 3.5, Fig. 3.8) and have various symbiotic properties on the five tropical legumes tested (Tables 3.2, 3.3).

Parasponia plants inoculated with the parent strain ANU280 produced nodules with no detectable acetylene reduction activity (Table 5.1). In all seventeen mutant strains tested, sixteen mutant strains which were similar to their parent strain ANU280 had a $Nod^{+}Fix^{-}$ phenotype on *P. andersonii* (Table 5.1). Only strain ANU2895 (Group 9), however, was found to induce nodules with a brown colour (Fig. 5.1 a, b) that were able to reduce acetylene (Table 5.1).

To confirm that this effective nodulation was not due to contamination, bacteria were re-isolated from these Fix^{+} nodules and tested for colony morphology, antibiotic resistant pattern, plasmid content, location of the transposon and nodulation ability. The re-

isolated bacteria had the same phenotype, genetic and physical markers as the inoculant strain ANU2895 and were able to effectively re-nodulate *P. andersonii* when they were re-inoculated on the plant. This indicates that mutant strain ANU2895 is capable of forming nitrogen-fixing nodules on the non-legume *P. andersonii*.

Effective nodulation of *P. andersonii* by strain ANU2895 was also observed using the beaker plant assay method. Similar results were obtained from different methods indicating that the formation of nitrogen-fixing nodules on *Parasponia* by the mutant strain ANU2895 is not conditional and dependant on the assay procedure.

5.3 COMPARISON OF NODULATION OF *PARASPONIA* BY STRAINS ANU280, ANU2895 AND CP279, A *PARASPONIA BRADYRHIZOBIUM* STRAIN

Nodule appearance on plants inoculated with strains ANU280, ANU2895 and CP279 was thirteen, twelve and seven days using the plate assay method. Six weeks after inoculation, parent strain ANU280 had nodulated 8% of *P. andersonii* plants but these were unable to fix nitrogen (Table 5.2). This is similar to the observation by Bender *et al* (1987a), using the same method. The nodulation frequency of strain ANU280 was significantly lower than that of the *Parasponia Bradyrhizobium* strain CP279 which effectively nodulated 100% of plants (Table 5.2) at the same time and under the same assay condition. The nodulation and nitrogen fixation capacity of strain ANU2895 on *Parasponia* was intermediate between that of strains ANU280 and CP279. The nodulation frequency of mutant strain ANU2895 was 28% which was 3.5-fold higher than its parent strain ANU280. About 75% of the nodulated plants produced nodules that were able to fix nitrogen at a

similar rate to the Fix$^+$ nodules formed by the *Parasponia* strain CP279 (Table 5.2).

The Fix$^+$ nodules formed by strain ANU2895 were isolated from different plants and embedded for analysis by light microscopy. Microscopic examination of nodule tissue showed a normal *Parasponia* nodule structure (Fig. 5.1 c, d).

5.4 DISCUSSION

Parasponia spp. are the only non-legume plants, so far found that are able to produce nitrogen-fixing nodules with certain *Rhizobium* strains (Trinick, 1973; Akkermans *et. al.*, 1978; Becking, 1979). The bacteria isolated from nodules on *Parasponia* species (*Parasponia Rhizobium*) are physiologically similar to slow-growing rhizobia (Trinick, 1976). Effective *Rhizobium* associations with *Parasponia* are obtained with only some slow-growing isolates from a number of tropical legumes (Trinick and Galbraith, 1980). Although many fast-growing *Rhizobium* strains from either tropical or temperate legumes have been found to be able to nodulate *Parasponia*, none were able to induce effective nitrogen-fixing nodules (Trinick and Galbraith, 1980; Becking, 1983). This indicates that the slow-growing *Rhizobium* from tropical legumes are closer to *Parasponia Rhizobium*.

The results presented in this chapter show that a single mutation of the genome of strain NGR234 enabled this fast-growing strain to form effective nitrogen-fixing nodules on *P. andersonii*. Strain ANU2895 is a Group 9 mutant that overproduces EPS. The mutation was induced by a Tn*5* transposon inserted into a 7.5kb *Eco*RI fragment (Table 3.5). The same mutation failed to induce nitrogen-fixing

nodules on four tropical legumes (Table 3.2) but was able to form nitrogen-fixing nodules on the non-legume plant, *Parasponia* (Table 5.1). This indicates: (a) that the genes required for effective nodulation of *Parasponia* are present but not expressed in strain NGR234 and; (b) that strain NGR234 can be made into a *Parasponia*-like *Rhizobium* by the appropriate mutation. These results suggest that the type or amount of EPS produced by strain NGR234 is important in host specific nitrogen fixation. The alteration in EPS synthesis can alter the nodulation phenotype of strain NGR234 not only from Nod^+Fix^+ to Nod^+Fix^- but also from Nod^+Fix^- to Nod^+Fix^+.

The reason why the mutant strain ANU2895 is able to effectively nodulate *Parasponia* is unclear. One possibility is that strain NGR234 is still incompatible with *Parasponia* and the 2895-mutation alters the bacterial surface to a more compatible structure or organization which masks the bacterium from recognition by the plant and hence it is not rejected. Such a proposed plant reaction would have to occur late in nodule development as the parent strain NGR234 can form nodules to an advanced stage on *Parasponia andersonii*.

Table 5.1 Plant response to exopolysaccharide-synthesis mutants

Strain	Mucoid group	Plant response	n mole C_2H_4/mg fresh nodule/hr
ANU280	Parent strain	Nod^+Fix^-	< 0.1
ANU2849	1	Nod^+Fix^-	< 0.1
ANU2885	1	Nod^+Fix^-	< 0.1
ANU2811	2	Nod^+Fix^-	< 0.1
ANU2820	2	Nod^+Fix^-	< 0.1
ANU2823	2	Nod^+Fix^-	< 0.1
ANU2831	2	Nod^+Fix^-	< 0.1
ANU2840	2	Nod^+Fix^-	< 0.1
ANU2842	2	Nod^+Fix^-	< 0.1
ANU2871	2	Nod^+Fix^-	< 0.1
ANU2814	5	Nod^+Fix^-	< 0.1
ANU2860	6	Nod^+Fix^-	< 0.1
ANU2899	7	Nod^+Fix^-	< 0.1
ANU2858	8	Nod^+Fix^-	< 0.1
ANU2833	9	Nod^+Fix^-	< 0.1
ANU2861	9	Nod^+Fix^-	< o.1
ANU2866	9	Nod^+Fix^-	< 0.1
ANU2895	9	Nod^+Fix^+	11.5

The plate assay method was used for the plant assay.

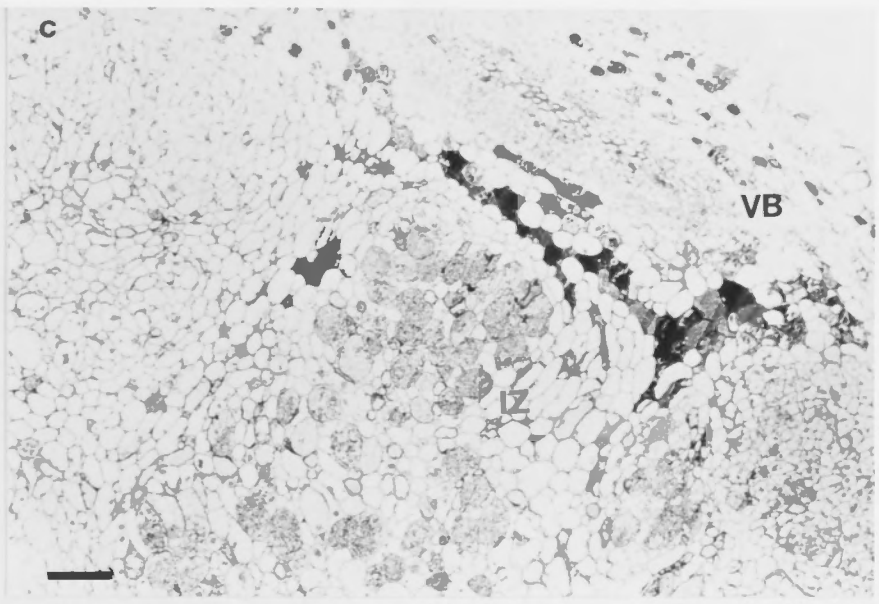
Table 5.2 Comparison in nodulation of *Parasponia andersonii* between fast-growing *Rhizobium* and *Parasponia Bradyrhizobium*

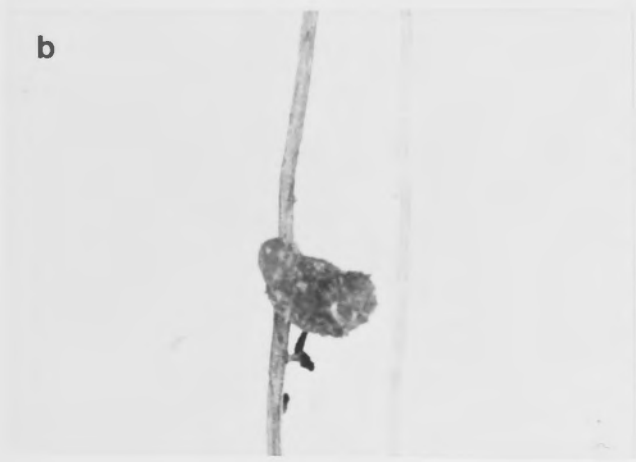
Strain	No. of Expts.	No. of plants tested	No. of plants nodulated	(%)	No. of nodulated plants Fix^+	(%)	n mole C^2H^4/mg fresh nodule/hr
ANU280	5	72	6	8	0	0	< 0.1
ANU2895	6	84	24	28	18	75	12
CP279	3	48	48	100	48	100	13

The plate assay method was used for the nodulation test.

Fig. 5.1. Nitrogen-fixing nodules induced on *Parasponia andersonii* by the mutant ANU2895. (a) Nitrogen-fixing plant on the plate six weeks after inoculation; (b) the effective nitrogen-fixing nodule; (c) sections through the Fix^+ nodule showing the vascular bundles (VB) and infection zone (IZ) and (d) the infection thread-like structure (T) packed with bacteria


a


c
VB
IZ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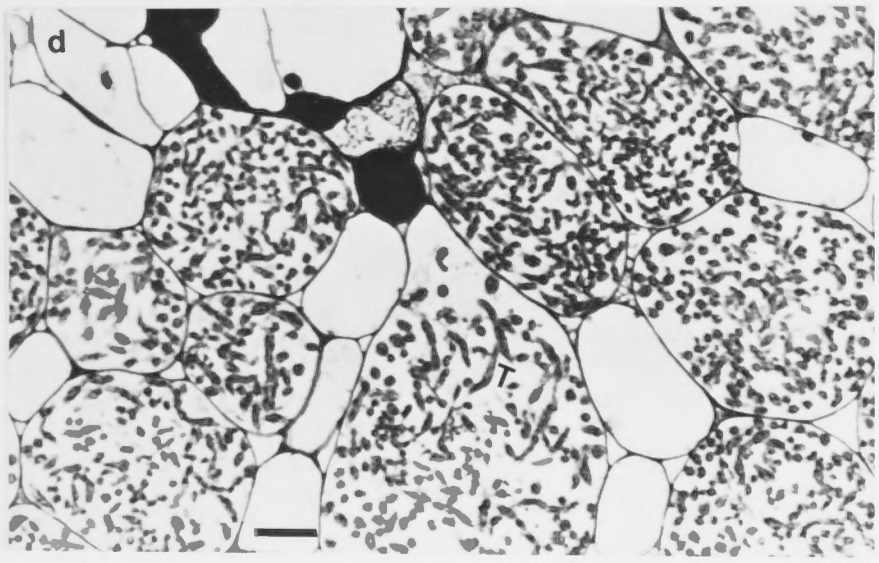
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T

CHAPTER SIX

INFECTION OF *LEUCAENA LEUCOCEPHALA* BY STRAIN ANU280

6.1 INTRODUCTION

Two types of *Rhizobium* infection occur in the nodulation of legumes. Firstly, bacteria may enter plant roots by forming infection threads in root hair cells (infection thread entry) as in *Macroptilium atropurpureum* (siratro) (Ridge and Rolfe, 1986). The threads penetrate the root cortex stimulating cell division; rhizobia are released by a breakdown of the infection thread wall. Alternatively, rhizobia may enter legume roots through a gap between epidermal cells at lateral root junctions ("crack entry") as observed in *Stylosanthes* nodulation (Chandler *et. al.*, 1982) . Rhizobia are distributed intercellularly and may invade the middle lamellea and enter the cortical cells through a structural alteration of the host cell wall. The *Rhizobium* induced nodule tissue is then formed by the repeated division of the invaded cells. It was noted that infection threads had not been found in root hairs or nodules for the tropical legume *Leucaena leucocephala* (Dart, 1977).

Rhizobium strain NGR234 isolated from the tropical legume *Lablab purpureus* resembles fast-growing rhizobia from *L. leucocephala* and is able to effectively nodulate *L. leucocephala* (Trinick, 1980 b; Morrison, 1984). Chapter Three showed that *L. leucocephala* was extremely sensitive in its response to exopolysaccharide-deficient (Exo^-) mutants of strain ANU280 (NGR234). Chapter Four demonstrated

that the ability of *Rhizobium* to produce EPS was fundamental to the induction of an effective nitrogen-fixing nodule on *Leucaena*. Furthermore, in Chapter Four infection thread-like structures were observed in large callus-like pseudonodules formed by some Exo^- mutants of strain ANU280 which suggested that bacterial infection of *L. leucocephala* was by infection thread entry. Thus, in this chapter the infection process of strain ANU280 in *L. leucocephala* has been examined in more detail using both light and electron microscopy with the view of establishing how *Rhizobium* surface polysaccharides might influence this infection process. The results demonstrate that *L. leucocephala* roots are infected via formation of infection threads in root hairs. Infection threads were also found in *Rhizobium* invaded cells within *Leucaena* nodules.

6.2 RESULTS AND DISCUSSION

6.2.1 *Root hair curling and hair deformation*

Strain ANU280 was used as the inoculum. Two different types of root hairs, short and long, were found on both tap and lateral roots. The long hairs (about 300-680 µm long) were often aggregated together on some of lateral junctions (Fig. 6.1 a) or elsewhere on the tap and lateral roots. These hairs did not deform in the presence of rhizobia. The short hairs (about 80-230 µm long) on inoculated plants showed curling or branching (Fig. 6.1 c) whereas no deformations were seen of short root hairs on uninoculated plants (Fig. 6.1 b).

Lateral roots first appeared three days after transferring two and a half day-old seedlings onto the agar medium. The majority of root

hairs were formed on lateral roots (Fig. 6.1 b). When seedlings were transplanted no root hair deformation was observed. The deformation of root hairs of inoculated plants was first observed 5 days after inoculation. The deformed root hairs almost always occurred on lateral roots (Fig. 6.1 c) but occasionally were observed on the tap root. Bacterial adhesion to the tip of the curled root hairs (Fig. 6.1 d) was occasionally observed.

6.2.2 *Infection threads in root hair*

It was very difficult to see infection threads in *Leucaena*. However, by careful examination of the root hairs of plants between ten to eighteen days after inoculation, a few infection threads were observed in the curled root hairs on lateral roots (Fig. 6.2 a). Figure 6.2 b shows an infection thread growing down into the hair cell. Infection threads were also examined by fixing and embedding curled root hairs. Light microscopy of serial sections of embedded root hairs showed infection threads in short, curled hairs (Fig. 6.2 c, d).

6.2.3 *Nodule distribution and infection threads in nodules*

Nodules were visible 12 days after inoculation, mostly on lateral roots (Fig. 6.3). Some nodules were located on the lateral roots very near to junction with the main tap root. Nodules were occasionally seen on the lower taproot. Nodules were not found at lateral roots junctions. A similar nodulation pattern was observed in *Leucaena* plants inoculated with *Leucaena Rhizobium* species (Trinick, 1965).

This suggests that in *L. leucocephala* the root hairs on lateral root are more infectible by *Rhizobium* than tap root cells. The reason for this phenomenon is unknown. Perhaps the root hairs on tap roots have different cell wall structures from that on lateral roots. Between 5 to 23 nodules were formed on each plant 4 weeks after inoculation which was more than the number of observed infection threads. The mature nodules were cylindrical and pink, having a persistent meristematic activity and an extensive bacterial zone containing rod shaped non-swollen bacteria (Fig. 4.2, Fig. 6.4 a). Infection threads were found between cells (Fig. 6.4 a) and entering cells packed with bacteroids (Fig. 6.4 b). Most of bacteroids were individually enclosed in a peribacteroid membrane (Fig. 6.4 c). These results indicated that *L. leucocephala* nodules develop from an infection of root hair by rhizobia.

6.2.4 *Effect of exopolysaccharide or oligosaccharide repeat-unit on root hair*

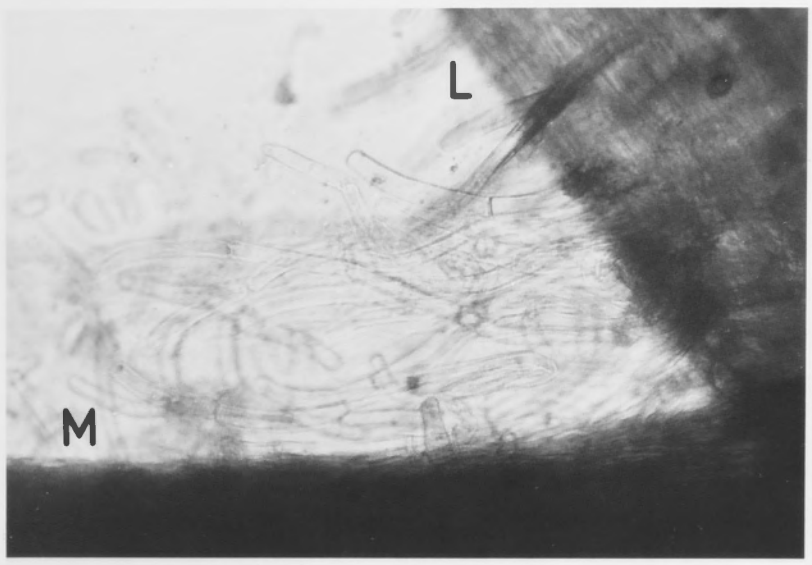
Purified EPS from *R. trifolii* can induce root hair deformation in white clover (Ervin and Hubbell, 1985). Exo^- mutants of *R. meliloti* were unable to induce root hair curling on *Medicago sativa* although they still formed nodules which did not contain infection threads (Leigh *et. al.*, 1985). The Exo^- mutants of strain ANU280 were still able to induce root hair deformation on *Leucaena* plants (Fig. 4.1) typical of that induced by the parent strain ANU280 (Fig. 6.1c) although they formed callus-like pseudonodules which either lacked infection threads or contained infection threads which did not release bacteria into plant cells (Figs. 4.3-4.5). Addition of purified EPS

from strain ANU280 or the oligosaccharide repeat-unit from the EPS to uninoculated *Leucaena* roots had no effect on root hairs. The EPS produced by strain ANU280 may not be needed for induction of root hair deformation but may be needed for the initiation or development of a proper infection thread in *Leucaena*.

6.3 ACKNOWLEDGEMENTS

R. W. Ridge and S. Z. Huang are thanked for help with microscopic examinations. Most of the results shown in this chapter will appear in the paper "*Rhizobium* infection of *Leucaena leucocephala* via the formation of infection threads in curled root hairs" by Hancai Chen and Barry G. Rolfe in Journal of Plant Physiology (in press).

Fig. 6.1 Root hairs on the roots of *L. leucocephala* seedlings. (a, top left) Long and (b, bottom left) short root hairs are located on both lateral (L) and main (M) roots of the uninoculated plant; (c, top right) only short root hairs were found curled or branched, mainly on the lateral roots of the inoculated plant; (d, bottom right) bacteria adhered to the tip of curled root hairs. The plants were examined seven days after transferring seedings onto the agar slope.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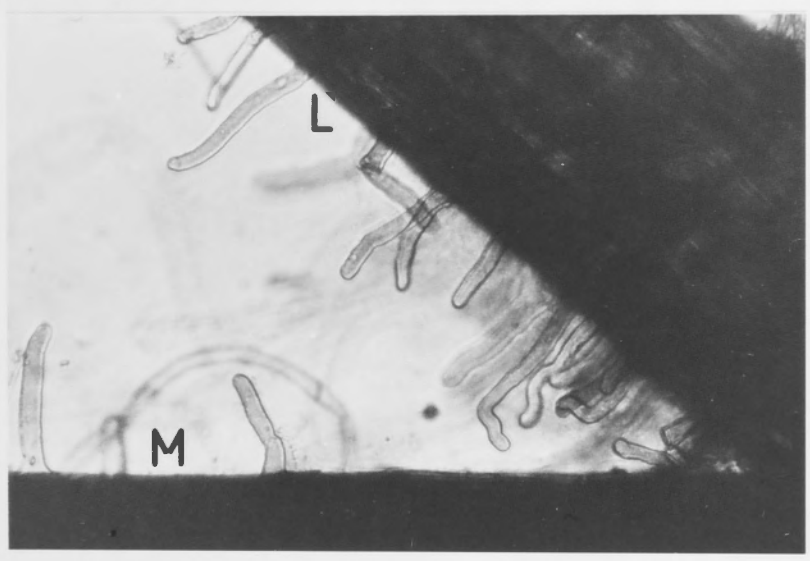
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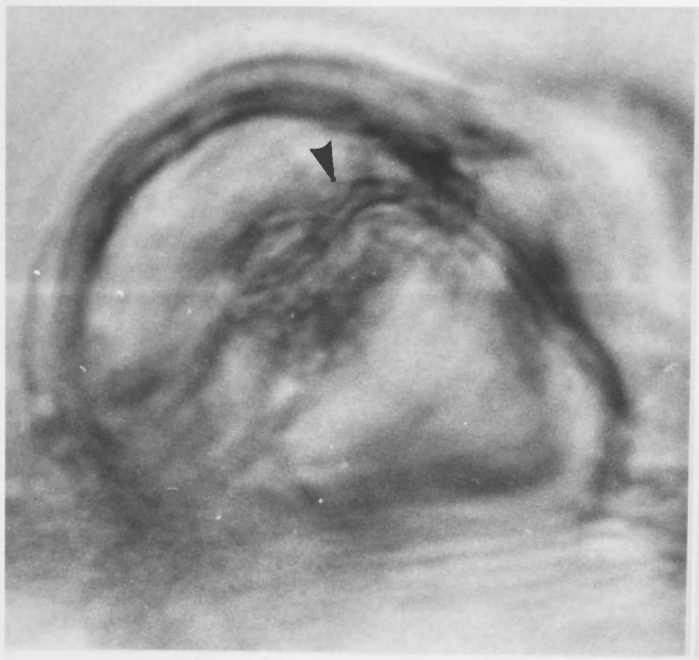
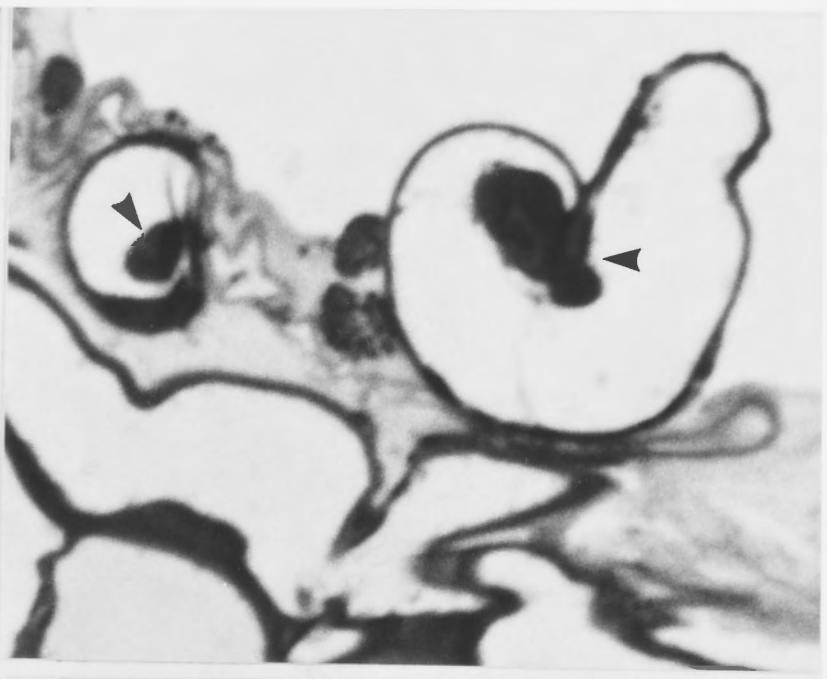
Fig. 6.2 Infection threads in curled root hairs. (a, top left) and (b, bottom left) Infection threads (arrow) in the intact curled root hairs 12 days after inoculation and; (c, top right) and (d, bottom right) light microscope sections of infection in curled root hairs 11 days after inoculation. Bar represents 20μm.





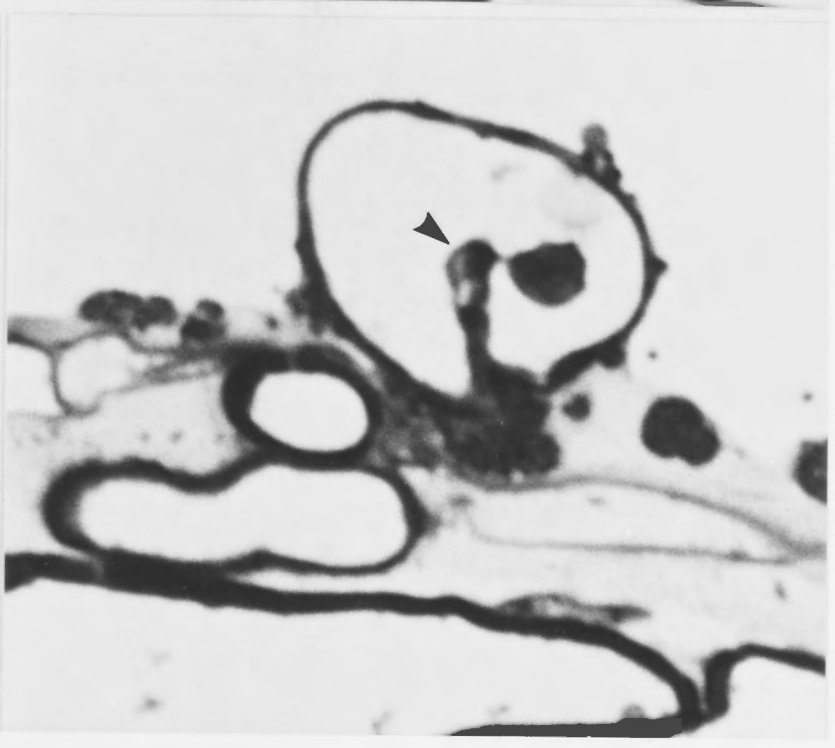


Fig. 6.3 Nodule location of *L. leucocephala*. Four weeks after inoculation nodules were mainly located on lateral roots.

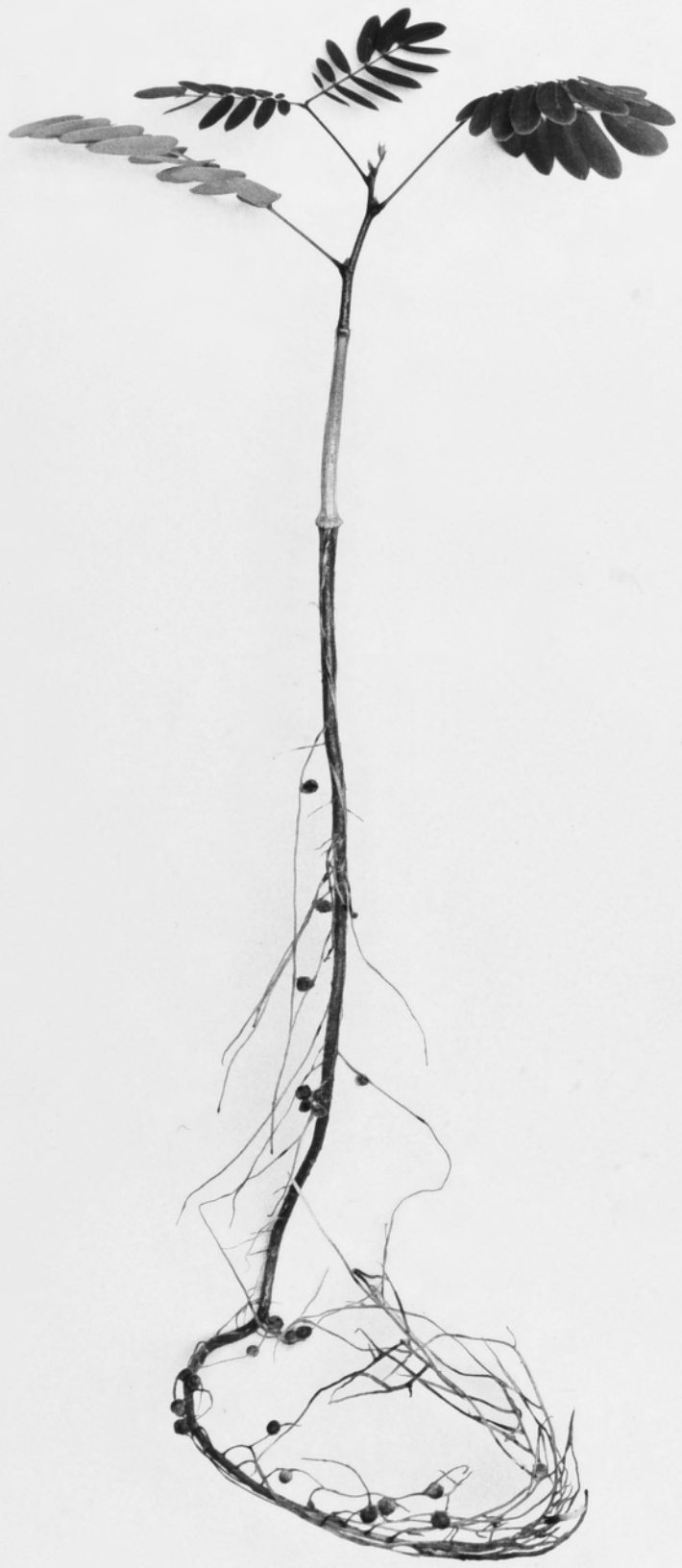
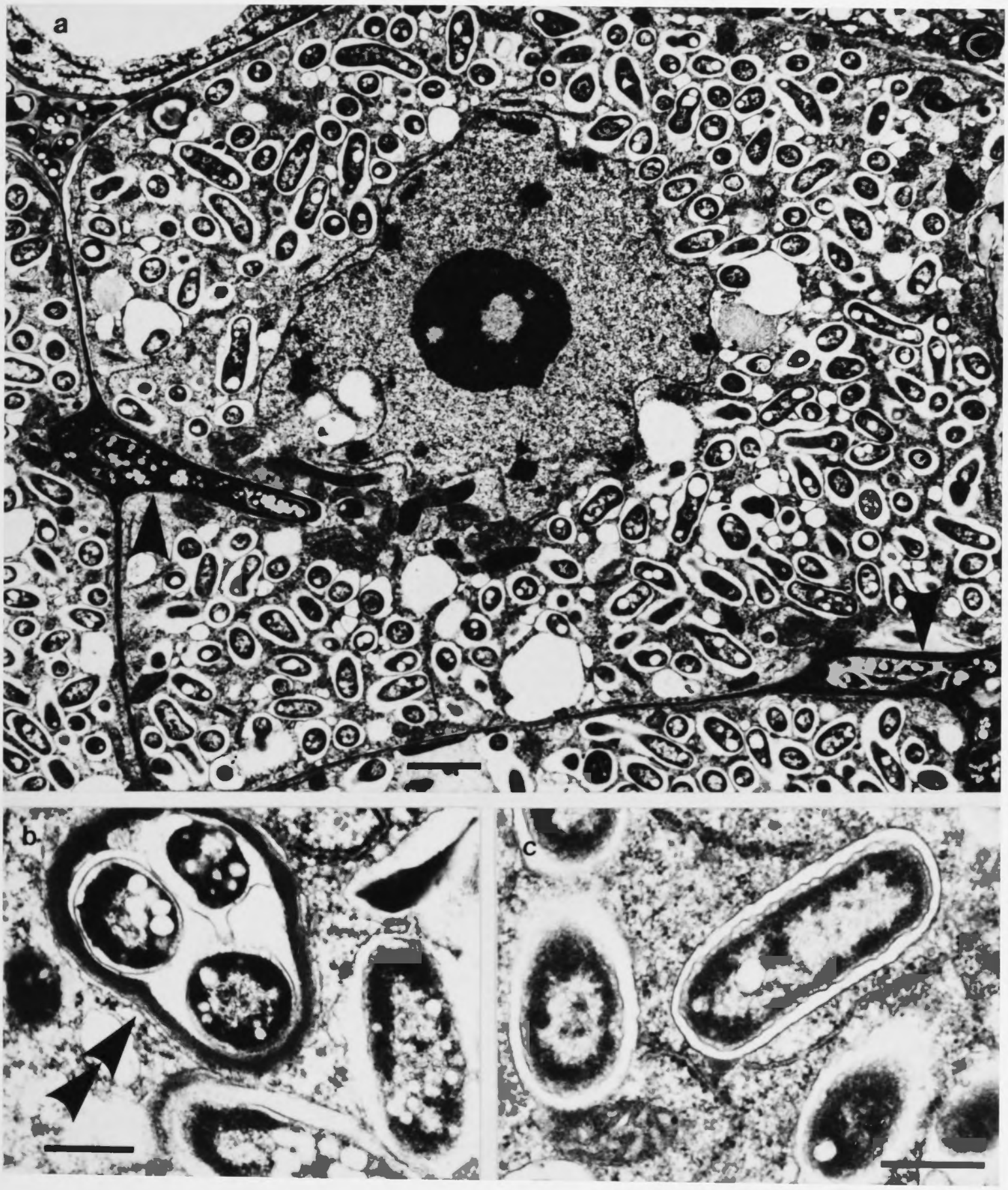


Fig. 6.4 Infection threads and bacteroids in electron microscope sections of an effective nodule 3 weeks after inoculation. (a) Intercellular (single arrow) and (b) intracellular infection threads (double arrows) in infected nodule cells filled with bacteroids; (c) bacteroid individually packed in a peribacteroid membrane. Bar represents 2μm in (a) and 0.5μm in (b) or (c).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CHAPTER SEVEN

RESTORATION OF NITROGEN-FIXING ABILITY OF EXOPOLYSACCHARIDE SYNTHESIS MUTANTS BY THE ADDITION OF PURIFIED EXOPOLYSACCHARIDE

7.1 INTRODUCTION

Chapter Three showed that the ability to synthesize large amounts of exopolysaccharides (EPS) is an important factor in the establishment of an effective symbiosis (Fix^+). The possibility of EPS being involved in the formation of an effective nitrogen-fixing nodule was further supported by mixing experiments which involved the mixing of a Muc^- Nod^+ (callus-forming) Fix^- strain with a Muc^+ Nod^- (Sym plasmid-cured) strain and inoculating *Leucaena* plants (Chapter Four). A cooperative interaction between the Muc^- Nod^+ Fix^- and the Muc^+ Nod^- mutant could lead to the formation of an effective nitrogen-fixing nodule on *Leucaena* plants (Table 4.1).

Several reports have presented evidence indicating that the purified or crude EPS could enhance the infectivity of *Rhizobium japonicum* (Baner *et. al.*, 1979) or *R. meliloti* (Olivares *et. al.*, 1984). So far, however, it has not been reported that the effective nitrogen-fixing nodules can be formed by adding purified or crude EPS to Muc^- Nod^+ Fix^- strains.

The EPS from *Rhizobium* strain ANU280 has been recently isolated and purified. The structure of this EPS which consists of a nonasaccharide repeat unit has been determined (Djordjevic S. *et.*

al.,1986). The EPS (s) of several Tn*5*-induced mucoid mutants have also been isolated and purified and their structures have been determined as well (Batley, Djordjevic S. and Redmond personal communication). In this chapter, the effect of purified EPS or its oligosaccharide repeat-unit from strain ANU280 on nodulation of plants by the exopolysaccharide-deficient (Exo^-) mutants has been tested. In addition, different EPS structures also were tried. The experiments in section 7.3 were done jointly with MR Steven Djordjevic and have been recently published (Djordjevic S *et. al.*, 1987a).

7.2 EFFECT OF THE ADDITION OF PURIFIED EPS OR RELATED OLIGOSACCHARIDE AND EXO^- MUTANTS TO *LEUCAENA*

Three Group 2 mutants (ANU2811, ANU2820 and ANU2840) were chosen as the representatives of Exo^- mutants. All of these strains have a single copy of transposon Tn*5* present in genomic DNA fragments (Table 3.5); fail to produce any detectable acidic oligosaccharide (Batley and Redmond personal communication) and have defective symbiotic phenotypes on different legume plants (Table 3.3). Furthermore, the inability of these Exo^- strains to induce a nitrogen-fixing nodule on *Leucaena* can be restored by co-inoculation with the Exo^+ Nod^- strain ANU265 (Table 4.1).

All the purified EPS (wild-type or mutant) and oligosaccharide were kindly supplied by Drs M. Batley and J. Redmond. The EPS isolated from strain ANU280 consists of nonasaccharide repeat units containing 5 glucose, 2 galactose and 2 glucuronic acid residues (Djordjevic S. *et. al.*, 1986). Purified EPS or related oligosaccharide isolated from strain ANU280 was inoculated onto

Leucaena plants together with one of the Exo$^-$ mutants. In all cases, the coinoculation of the EPS or the oligosaccharide repeat-unit enabled the Exo$^-$ mutants to induce nitrogen-fixing nodules on *Leucaena* plants, although some calli were still produced (Fig. 7.1, Table 7.1). The same behaviour was observed whether the EPS was obtained from strain ANU280 or ANU265. To confirm that the nitrogen-fixing nodules formed were due to the addition of EPS rather than Exo$^+$ revertants, the bacteria were re-isolated from 30 pigmented *Leucaena* nodules (Fix$^+$) and tested for their phenotypic properties. Bacterial isolates from these Fix$^+$ nodules still maintained their original phenotype and symbiotic properties, indicating that the effective nodulation abilities of strains ANU2811, ANU2820 and ANU2840 were due to the addition of the purified EPS from strain ANU280.

7.3 EFFECT OF THE ADDITION OF EPS AND EXO$^-$ STRAINS TO SIRATRO PLANTS

Parallel experiments like those described above for *Leucaena* plants were done on siratro plants where the parent strain induces nitrogen-fixing determinate nodules and the Exo$^-$ mutants ANU2811 and ANU2820 alone induce normal-looking nodules which fail to fix nitrogen. The addition of EPS from strain ANU280 24 hours prior to inoculation with the Exo$^-$ strain ANU2811 or ANU2820 enabled these bacteria to produce determinate nitrogen-fixing nodules on siratro plants like the parent strain (Table 7.2).

7.4 EPS FROM MUTANT STRAIN ANU2858

Strain ANU2858 is a Group 8 mutant which produces about 10% of wild-type levels of EPS (Table 3.4) and forms ineffective, small nodules on siratro, *Desmodium intortum, D. uncinatum, Lablab purpureus* and *L. leucocephala* (Table 3.2). Chemical analysis has shown that the structure of the EPS produced by mutant ANU2858 is very different from the structure of the EPS produced by the parent strain ANU280. The EPS from mutant ANU2858 consists of tetrasaccharide repeat units containing 2 rhamnose, 1 methylated rhamnose and 1 methylated glucuronic acid residues (Djordjevic S. personal communication). To determine whether there is a correlation between the structure and function of EPS, the purified EPS from ANU2858 was added to ANU2840 which was used to inoculate *Leucaena* plants. The plants inoculated with ANU2840 together with the EPS of ANU2858 were unable to produce a nitrogen-fixing nodule (Table 7.1) indicating that the function of EPS is related to its structure. Furthermore, the EPS of ANU280 was able to enhance the nodulation ability of ANU2858 to induce a larger but non-nitrogen-fixing nodule on siratro while the EPS of ANU2858 did not affect the nodulation ability of ANU2858 (Table 7.2). This indicated that the inability of ANU2858 to form a nitrogen-fixing nodule was connected with its inability to produce the normal levels and structure of wild-type EPS.

7.5 EPS FROM MUTANT ANU2861

Strain ANU2861 is a Group 9 mutant which over-produces EPS (forming up to 220% more EPS compared with its parent strain ANU280)

and forms very slimy, translucent colonies on Mannitol medium (Table 3.1). This strain is unable to nodulate siratro, *Desmodium*, and to form a non-nitrogen-fixing nodule on *Lablab* and *Leucaena* (Table 3.2). On siratro, it induces a hypersensitive-like plant response (Figs. 3.4-3.6). When *Leucaena* plants were inoculated with ANU2840 together with purified EPS from ANU2861, the plants were able to produce nitrogen-fixing nodules (Table 7.1). However, when the EPS from ANU280 was added to strain ANU2861, it did not affect the nodulation ability of ANU2861 on siratro plants (Table 7.2).

7.6 PRE-TREATMENT OF *LEUCAENA* WITH PURIFIED EPS

A pre-treatment of plant roots with EPS from strain ANU280 was used to test whether the effect of added EPS to plants could persist. This was done by adding the purified EPS from strain ANU280 to the roots of *Leucaena*, and after 24 hours or 48 hours the EPS was washed from the roots. Then these plants were inoculated with the Exo^- mutant ANU2840. The results in Table 7.3 show that the plants with 24 hour pre-treatment produced ineffective nodules and the plants with 48 hour pre-treatment produced effective nodules.

7.7 DISCUSSION

Results in this study demonstrate unambigously that restoration of the Fix^+ phenotype can be achieved by the addition of homologous EPS or oligosaccharide from the appropriate parent strain. Restoration of the Fix^+ phenotype was successful with both indeterminate (*Leucaena*) and determinate (siratro) nodule forming plants.

The effect of EPS on plants may be an active process which occurs early before the infection of the root and affects the subsequent stages of nodulation. The evidence supporting this possibility was: (a) that Exo^- mutants could effectively nodulate *Leucaena* plants which were pre-treated by EPS for 48 hours, (b) that the function of EPS was related to its structure and (c) that heterologous saccharides failed to correct the defective symbiotic phenotype (Djordjevic S *et. al.*, 1987). Recently, specific oligosaccharides derived from the cleavage of host or plant pathogen cell walls have been postulated as regulators of specific plant functions, such as growth, differentiation and disease resistance (Darvill and Albersheim, 1984; Keim *et. al.*, 1985). It is possible that the EPS or part of it functions similarly. The action of EPS may depend on the presence of plant enzymes (Bhagwat and Thomas, 1984; Dazzo *et. al.*, 1982; Solheim and Fjellheim, 1984) which degrade the bacterial polymer to active oligomers which have a specific role in effective nodule formation.

The EPS from the wild-type strain ANU280 could enhance the nodulation ability of mutant ANU2858 to form larger nodules on siratro but could not restore the effective symbiotic ability of the bacteria. This may be due to the presence of the structurally altered mutant EPS which might inhibit an effective nodulation. This observation is very interesting, because it may be possible to see different plant responses by mixing different exopolysaccharides in different ratios to treat plants, or by treating plants with different exopolysaccharides at different times.

The EPS from mutant ANU2861 could correct the defective symbiotic phenotype of Exo^- mutants, suggesting that the ability of this strain to induce hypersensitive-like plant response (Figs. 3.4-3.6) is not

related to its EPS. This is consistent with the results from chemical analysis that the structure of the EPS produced by mutant ANU2861 is identical to that produced by the parent strain ANU280 (Redmond and Batley personal communication). Ridge (1985) and Djordjevic S. (1987) have found that mutant ANU2861 is not only able to initiate a rapid hypersensitive type reaction at the site of infection, but also seems to be able to induce a systemic response in the roots of siratro plants (Rolfe *et. al.*, 1986).

The generality of the correction phenomenon remains to be demonstrated. While the results in this study imply that it would be possible to use different polysaccharides to trigger the plant responses and thereby to find the substances produced by plants which can interact with the corresponding polysaccharides.

7.8 ACKNOWLEDGMENTS

Thanks to Drs. Micheal Batley and John W. Redmond for providing EPS and oligosaccharide. Results in sections 7.2 and 7.3 have appeared in the paper "Nitrogen-fixing ability of exopolysaccharide synthesis mutants of *Rhizobium* sp strain NGR234 and *R. trifolii* by the addition of homologous exopolysaccharide" by Steven P. Djordjevic, Hancai Chen, Michael Batley, John W. Redmond and Barry G. Rolfe in Journal of Bacteriology, volume 169: 53-60 (1987).

Table 7.1 Effect of adding purified EPS or nonasaccharide repeat unit on *Leucaena* nodulation*

Strain inoculated and addition	Plant nodulation response	No. of plants tested	Percent of plants forming Fix^+ nodules
ANU280 (parent strain)	Large Fix^+ nodules and occasional small calli	100	100
ANU2811	Small calli	100	2
ANU2840	Small calli	100	1
ANU2820	Small calli	20	0
ANU2858	Small Fix^- nodules	20	0
ANU2861	Small Fix^- nodules	20	0
ANU2811 + (EPS from ANU280)	Large Fix^+ nodules and small calli	30	35
ANU2811 + (oligosaccharide from ANU280)	Large Fix^+ nodules and small calli	20	30

Table 7.1 Continued

ANU2840 + (EPS from ANU280)	Large Fix^+ nodules and small calli	100	40
ANU2840 + (oligosaccharide from ANU280)	Large Fix^+ nodules and small calli	20	40
ANU2840 + (EPS from ANU2858)	Small Fix^- nodules and small calli	20	0
ANU2840 + (EPS from ANU2861)	Large Fix^+ nodules	20	20
ANU2820 + (EPS from ANU280)	Large Fix^+ nodules and small calli	20	25
ANU2820 + (oligosaccharide from ANU280)	Large Fix^+ nodules and small calli	10	30

* Nitrogen-fixing nodules induced by the addition of EPS or oligosaccharide repeat unit and Exo^- mutants on *Leucaena* reduce acetylene 20 to 70% as effectively as Fix^+ nodules induced by the parent strain, ANU280. Percentages of plants forming Fix^+ nodules are averages of ten plant batch experiments. In each batch of 10 plants, the percentage of plants forming Fix^+ nodules varied from 20 to 80% with each Exo^- mutant. EPS or oligosaccharide repeat unit (5µl of 5mg ml^{-1}) was added 24 hours prior to inoculation with Exo^- bacteria.

Table 7.2 Effect of adding purified EPS or oligosaccharide repeat unit on siratro nodulation*

Strain inoculated and addition	Plant nodulation response	No. of plants tested	Percent of plants forming Fix^+ nodules
ANU280 (parent strain)	Large Fix^+ nodules	250	>90
ANU2811	Small Fix^- nodules	80	2
ANU2811 + (EPS from ANU280)	Large Fix^+ nodules	40	40
ANU2820	Small Fix^- nodules	20	0
ANU2820 + (EPS from ANU280)	Large Fix^+ nodules	30	40
ANU280 + (oligosaccharide repeat unit from ANU280)	Large Fix^+ nodules	20	40
ANU2858	Small Fix^- nodules	40	0
ANU2858 + (EPS from ANU280)	Large Fix^- nodules	30	0
ANU2858 + (EPS from ANU2858)	Small Fix^- nodules	20	0
ANU2861	No nodules	40	0
ANU2861 + (EPS from ANU280)	No nodules	20	0

* EPS or oligosaccharide repeat unit (5µl of 5mg ml^{-1}) was added 24 hours prior to inoculation with Exo^- bacteria.

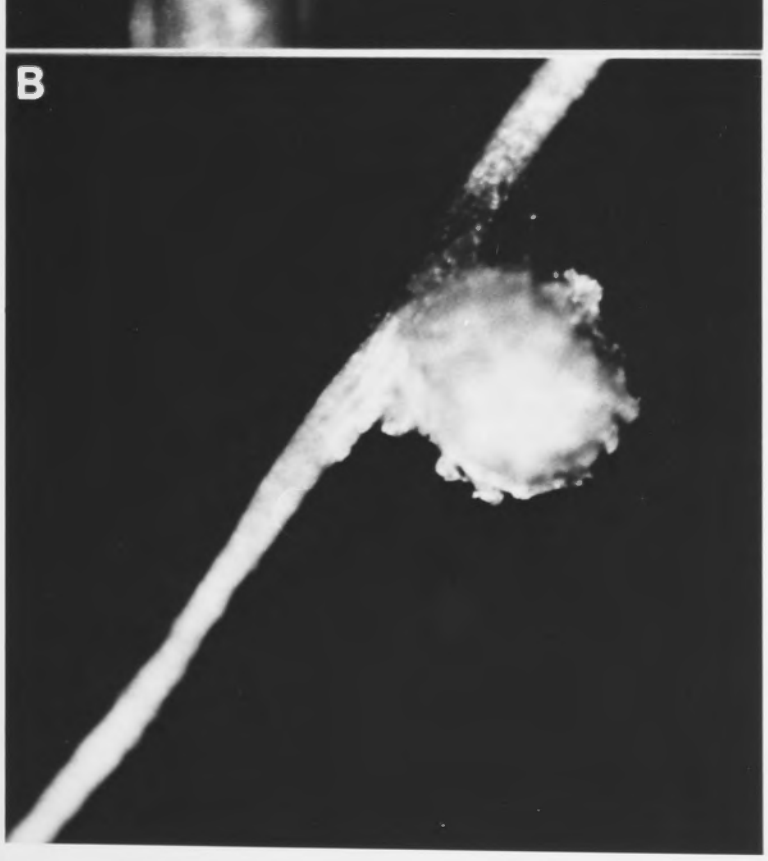
Table 7.3 Effect of pre-treating *Leucaena* with purified EPS from ANU280 on nodulation of *Leucaena* by ANU2840

Plant treatment#	Nodulation
No treatment	Calli
Added EPS 24h before inoculation	Fix^+ nodules
Added EPS 24h before inoculation, then washed roots and inoculated	Fix^- nodules
Added EPS 48h before inoculation	Fix^+ nodules
Added EPS 48h before inoculation, then washed roots and inoculated	Fix^+ nodules

EPS (5µl of 5mg ml^{-1}) was added 24 or 48 hours prior to inoculation with the Exo^- bacteria. The EPS was washed from plants by rinsing the plant roots with 20 glass pipettes of sterile F liquid medium.

Fig. 7.1 Responses of *Leucaena* plants to the addition of EPS or oligosaccharide repeat unit with Exo^- strains. (A) A nitrogen-fixing (C_2H_2-reduction) nodule induced by the parent strain ANU280; (B) non-nitrogen-fixing, distorted, callus-like nodule induced by the Exo^- mutant ANU2840; (C) ultrastructure of the Fix^+ nodule induced by ANU280; (D) the callus induced by mutant ANU2840 and; (E) the Fix^+ nodule induced by mutant ANU2840 after the addition of EPS from ANU280.


A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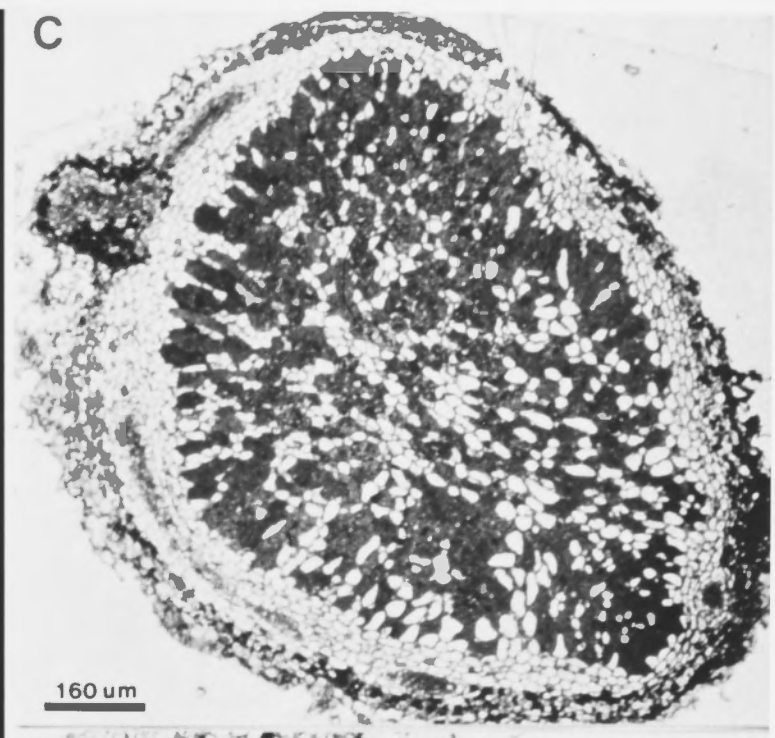
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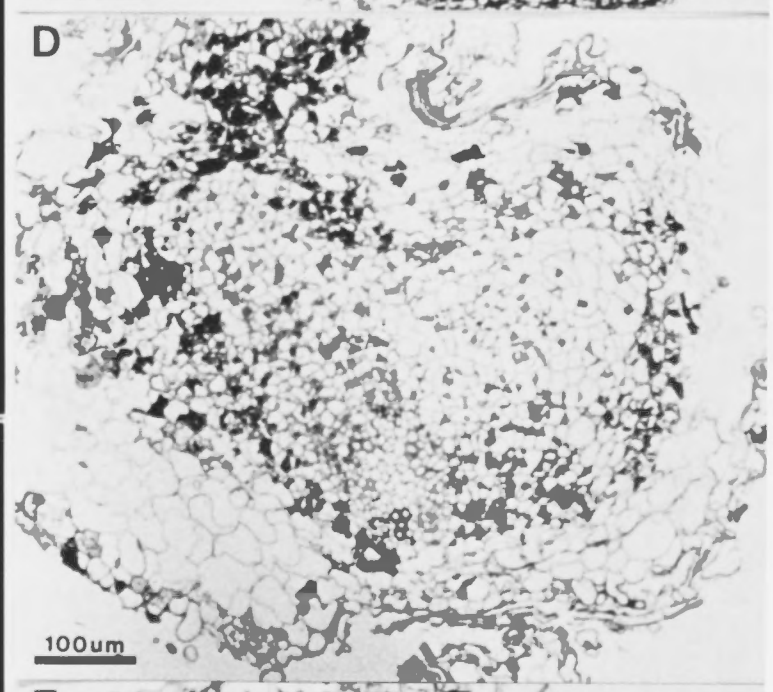
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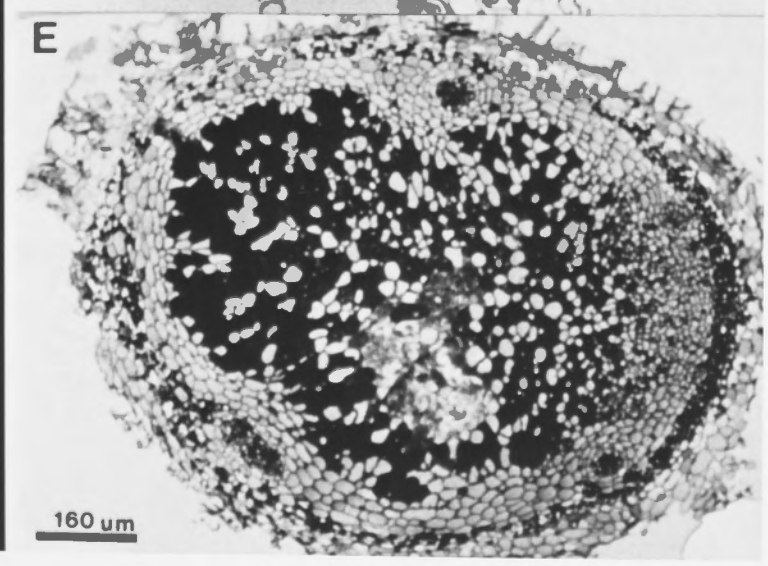
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CHAPTER EIGHT

ISOLATION OF R-PRIME PLASMIDS CARRYING REGIONS INVOLVED IN EXOPOLYSACCHARIDE SYNTHESIS OF *RHIZOBIUM* STRAIN NGR234

8.1 INTRODUCTION

Bacterial exopolysaccharide synthesis is controlled by a complex interaction of substrate uptake, intermediary metabolism and polymer synthesis (Sutherland, 1979). Many genes are involved in this complex process. Complementation of Exo$^-$ mutants of *R. meliloti* with the recombinant plasmids from the *R. meliloti* cosmid clone bank has shown that at least 11 different loci are responsible for the *Rhizobium* EPS synthesis and regulation (Leigh *et. al.*, 1985, 1986; Reed *et. al.*, 1986; Walker *et. al.*, 1986). Some of these loci have been mapped on a second megaplasmid which is distinct from the *nod-nif* megaplasmid while the others are located on the chromosome (Hynes *et. al.*, 1986; Finan *et. al.*, 1986), indicating that *Rhizobium* EPS genes are not clustered to a small region. Chapter Three showed that most of the *Tn5*-induced EPS mutants of strain NGR234 have resulted from the insertion of Tn*5* at different locations. This suggests that there should be many genes involved in EPS biosynthesis and regulation in strain NGR234.

To investigate the regulation of polysaccharide synthesis in strain NGR234 and the organization of genes involved in EPS production, the strategy adopted in this chapter was to isolate

R-prime plasmids carrying DNA regions of the strain NGR234 genome involved in polysaccharide synthesis. The advantages of R-primes constructed with plasmid R68.45 are: (a) they usually carry large regions of *Rhizobium* DNA (Banfalvi *et. al.*, 1983; Nayudu and Rolfe, 1987) which would be very useful in complementation analysis of previously isolated polysaccharide mutants of strain NGR234 to determine their genetic linkage and; (b) to enable the transmission of these polysaccharide genes to different bacterial species to determine if genes for exopolysaccharide synthesis are functionally conserved. This chapter describes the isolation of R-prime plasmids carrying DNA regions of strain NGR234 which play a role in polysaccharide synthesis. These R-primes were isolated from intergeneric matings between *Rhizobium* and *E.coli* cells using a kanamycin sensitive R68.45-type plasmid (Nayudu and Rolfe, 1987). It has been found that R-primes carrying a mutant Exo^- allele (Tn*5* insertion) which led to a marked reduction in polysaccharide synthesis had a dominant effect on the regulation of normal EPS biosynthesis in parent strain NGR234 and strains of *R. meliloti* and fast-growing *R. japonicum*.

8.2 ISOLATION OF R-PRIME PLASMIDS

A Group 2 Exo^- mutant of strain ANU280, strain ANU2811, was used to construct R-prime plasmids carrying genes involved in EPS biosynthesis. To select R-prime plasmids, plasmid pMN2 (a kanamycin-sensitive R68.45-like plasmid showing enhanced chromosome mobilizing ability) was transferred into mutant ANU2811. The resulting transconjugant was used as a donor in mating with *E.coli recA* strain HB101 selecting for the transfer of kanamycin resistance. Km^r

colonies occurred at a low frequency (approximately 1 x 10^{-7} per donor cell) and when repurified and tested, all were found to be tetracycline and carbenicillin resistant (pMN2 markers). Most of the Km^r transconjugants were able to co-transfer kanamycin resistance at 100% frequency when selection was imposed for the transfer of tetracycline resistance to a HB101 Rif^r derivative. This showed that the Km^r of Tn*5* is genetically linked to vector plasmid pMN2. Isolation and visualization of the putative R-prime plasmids using a modified Eckhardt technique (Plazinski *et. al.*, 1985; see Fig. 8.1) showed that the sizes of the plasmids present in these strains were far greater than pMN2 (67 kb) with the addition of Tn*5* (5.7 kb). This suggests that these plasmids carry different amounts of *Rhizobium* DNA sequences flanking the Tn*5* insertion and therefore are R-primes. By using this genetic technique, a family of R-prime plasmids was isolated. Several of these R-primes designated R'2811-1, R'2811-2, R'2811-8, and R'2911-9 were chosen for further characterization.

8.3 PHYSICAL CHARACTERIZATION OF R-PRIME PLASMIDS

Several approaches were used to determine the amount of DNA flanking the Tn*5* insertion point in the R-prime molecules. Restriction enzyme (*Eco*RI, *Cla*I, *Sma*I) analysis of each R-prime plasmid showed: (a) that different amounts of DNA were present in each plasmid and; (b) that many bands of similar size occurred in each of the digests (Figs. 8.3, 8.6). The size of the restriction fragments obtained on agarose gels was determined. Due to the large size of the DNA inserts, it was only possible to approximately estimate their sizes. Comparison of R-primes with the parent plasmid pMN2 (Figs.

8.3, 8.6) indicated that the sizes of the DNA inserts were 75kb (R;2911-1), 86kb(R'2811-2), 59kb(R'2811-8) and 64kb(R'2811-9). To confirm that much of the DNA contained in the R-prime plasmids was identical, a hybridization probe made from plasmid pKan2 or R'2811-9 DNA was used to probe DNA isolated from each of the four different R-prime molecules as well as total DNA from the parent mutant *Rhizobium* strain ANU2811. An analysis of the obtained hybridization patterns showed: (a) that each R-prime plasmid contains a single copy of Tn*5* located on a similar sized restriction fragment on the R-prime as in the mutant ANU2811 (Fig. 8.2); (b) that an overlapping region of about 52kb was present in all the R-prime molecules and; (c) that an identical hybridization pattern was obtained in the total DNA of the parent mutant strain. This suggests that the region around the Tn*5* insertion is unaltered in the R-prime and the DNA present represents a contiguous segment of *Rhizobium* DNA.

Digestion of vector plasmid pMN2 with *Sma*I produced a 2.1 kb fragment (Fig. 8.3) which have been shown to contain a tandem duplication of a pre-existing copy of IS*21* (Riess *et. al.*, 1980; willetts *et. al.*, 1981). If foreign DNA is inserted between the two copies of *IS*21 [as has been shown for other R68.45-prime plasmids by Leemans *et. al.* (1980)] then these two DNA fragments should not be present in the R-prime molecules. In the case of plasmids R'2811-1 and R'2811-9 the 2.1 kb *Sma*I fragment was absent however, in plasmids R'2811-2 and R'2811-8 a similar sized *Sma*I fragment was present (Fig. 8.3). When plasmid DNA was digested with *Cla*I, a 2.1 kb fragment was present in plasmid pMN2 but was absent in all of the four R-prime plasmids (Fig. 8.6). This suggested that the similar sized *Sma*I fragment in R'2811-2 and R'2811-8 was *Rhizobium* DNA rather than vector

pMN2 DNA. These results show that there has been a disruption of the tandem IS*21* duplication region on the R-primes indicating that the foreign DNA has inserted in this region.

8.4 ANALYSIS OF R-PRIMES IN THE PARENT STRAIN ANU280

With the view of doing complementation assays with the mutated R-prime derivatives, these mutated plasmids were introduced firstly to strains ANU280 and ANU2811. As expected, the transfer of the R-prime derivatives to ANU2811 resulted in Exo^- colonies however, an unexpected result occurred with the introduction of any of the R-prime plasmids to the parental (Exo^+) strain ANU280. All the transconjugant colonies in this instance possessed an Exo^- phenotype identical to the original phenotype of Exo^- strain ANU2811 (Fig. 8.4). The same result occurred if the introduced plasmid was: (a) transferred to the Sym plasmid cured derivative of strain NGR234 (strain ANU265) or (b) if passaged from an Exo^- transconjugant colony to *E.coli* strain HB101 and back to *Rhizobium* strain ANU280. The Km^r marker was co-transferred with the other plasmid markers in these matings. Moreover, the ANU280 Exo^- transconjugants possessed the same symbiotic defectiveness on *Leucaena leucocephala* plants as the original Exo^- strain ANU2811 [(ineffective callus-like growths compared to nitrogen-fixing nodules induced by the wild-type strain ANU280 (Table 3.3)]. This indicated that the 2811-Tn*5* mutation was dominant in the wild-type strain ANU280 background and that the Exo^- phenotype of the ANU280 transconjugants was stable since no evidence of the ability of this strain to produce nitrogen-fixing nodules was observed even at a low rate.

8.5 STABILITIES OF R-PRIME PLASMIDS

Despite containing a large segment of *Rhizobium* DNA, no marked instability of any of the R-primes was observed in *Rhizobium*. The stability of the R-primes in strain ANU280 was further tested by growing the transconjugant, ANU280(R'2811-9) under non-selective conditions for about 500 generations using a replica-plating technique. The Exo^- phenotype was retained in more than 99% of the colonies. All tested colonies contained a plasmid with genetic and physical properties the same as plasmid R'2811-9, indicating a high degree of stability of the R-primes in the parent strain ANU280 background.

8.6 CONSTRUCTION OF R-PRIMES CARRYING WILD-TYPE *RHIZOBIUM* DNA SEQUENCES

Complementation assays with either of the mutated R-primes were impossible considering the dominance effect caused by the introduction of these plasmids. However, transfer of either of these dominant, mutated R-primes to several of the Group 2 Exo^- mutants by selecting for Tc^r transconjugants, resulted in a small number of Exo^+ revertants which occurred at a frequency of about 10^{-6}-10^{-7}. One possibility is that the R-primes in these Exo^+ colonies could have lost their dominant 2811-Tn*5* allele due to recombination with homologous genomic sequences or alternatively the mutated allele could have been lost by deletion. To test this, the Exo^+ colonies were used as donors in matings with the *E.coli recA* strain HB101, selecting for the transfer of Tc^r. Only the *E.coli* transconjugants which were Tc^r and Km^s were

tested further as probable R-prime plasmids containing wild-type sequences. Three examples of $Tc^{r}Km^{s}$ HB101 transconjugants from each mating were then used as donor strains in matings with strains ANU280 and ANU2811. For the following reasons it was clear that the R-primes carried wild-type genomic sequences: (a) transfer of the Km^{s} R-primes to either ANU280 or to ANU2811 resulted in Exo^{+} transconjugants (complementation of the ANU2811 mutation); (b) restriction digests showed that the R-primes contained all of the restriction fragments of the original mutated R-primes except that the Tn*5*-containing band had disappeared and was replaced by a smaller band (Fig. 8.6); (c) no hybridization could be detected to a Tn*5*-specific probe in the putative wild-type R-prime derivatives and; (d) a hybridization probe (plasmid pE2811, see section 2.2.13c) which contained the ANU2811 allele and surrounding *Rhizobium* DNA sequences hybridized to an 8.1kb *Cla*I fragment in the mutant R-primes but only a 2.4kb *Cla*I fragment in the wild-type R-prime DNA.

Using this strategy, four different R-primes suspected of carrying wild-type DNA sequences of strain NGR234 were isolated. These R-primes were named R'3201 (derived from R'2811-1), R'3205 (derived from R'2811-2), R'3211 (derived from R'2811-8) and R'3222 (derived from R'2811-9). Plasmid pJG11, which was made by MR James X. Gray and contains a 10kb *Bam*HI fragment of DNA encompassing the 2811-Tn*5* insertion site sub-cloned from R'3222 into plasmid pUC18, was used as a radioactive probe to examine DNA homology between Km^{r} and Km^{s} R-prime plasmids and genomic DNA from strains ANU280 and ANU2811. This experiment was done jointly with James X. Gray. The hybridization patterns of the Km^{r} R-primes and their Km^{s} derivatives, along with the genomic DNA for ANU280 and ANU2811 were all identical except for the

fragment where the Tn*5* had inserted (Fig. 8.7). In strain ANU2811 the Tn*5* had inserted into a 0.6kb *Eco*RI restriction fragment and thus increased the size of this fragment to 6.3kb. These results demonstrated that R-prime plasmids R'3201, R'3205, R'3211 and R'3222 carry the wild-type sequences of the parent strain ANU280.

8.7 EFFECT OF R-PRIMES ON EPS PRODUCTION AND NODULATION ABILITIES OF MUTANTS ANU2861 AND ANU2866

R-prime plasmid R'2811-9 and its Km^S derivative R'3222 were individually transferred into two Group 9 mutants ANU2861 and ANU2866 (over-production EPS, *ade*$^-$, see section 3.4). The transconjugants carrying R'2811-9 had an Exo^- colony morphology while the colony phenotype of the transconjugants containing R'3222 had not changed. However, both R-prime plasmids did not correct the *ade*$^-$ phenotype of mutant ANU2861 or ANU2866. A plasmid which contains a 6.2kb *Eco*RI fragment encompassing the 2861-Tn*5* insertion site (Djordjevic S. 1987) was used as a radioactive probe to examine R'2811-9 and R'3222 for homologous sequences. The absence of any hybridization indicated that the 2861 locus is not located on the R-prime. Mutants ANU2861 and ANU2866 had a Nod^- phenotype on siratro and *Desmodium* plants (Table 3.2, Fig. 8.5 b). However, both Exo^- transconjugants [ANU2861(R'2811-9) and ANU2866(R'2811-9)] were able to nodulate these plants (Fig. 8.5 c). The nodules formed by the Exo^- transconjugants were small and non-nitrogen-fixing which were similar to the nodules formed by the mutant strain ANU2811 (Table 3.3). The transconjugants carrying R'3222 nodulated neither siratro (Fig. 8.5 d) nor *Desmodium* plants.

8.8 EXPRESSION OF R-PRIME PLASMIDS IN DIFFERENT *RHIZOBIUM* STRAINS

To investigate whether the dominant mutation carried on these R-primes could be expressed in different bacterial strains, plasmid R'2811-9 was transferred into different bacterial strains; *R. meliloti* strain 1021, fast-growing *R. juponicum* strain USDA191, *R. trifolii* wild-type strain ANU843 and the Sym plasmid-cured strain ANU845, *R. leguminosarum* Sym plasmid-deleted strain 6015 and *Agrobacterium tumefaciens* strain C58 and non-Ti-plasmid strain A136 by selecting for Km^r. The results in Table 8.1 show that the R-prime carrying the dominant mutation was able to inhibit EPS synthesis of strains 1021 and USDA191 but unable to affect the mucoid phenotype of strains ANU843, ANU845, 6015, C58 and A136. Plasmid R'2811-9 could be re-transferred from transconjugants ANU843(R'2811-9), ANU845(R'2811-9), 6015(R'2811-9), C58(R'2811-9) and A136(R'2811-9) via strain HB101 to strain ANU280 without loss of either Km^r and Tc^r markers or the dominance effect. This indicates that the R-prime plasmids were stable in these different backgrounds and lack of visual effect was not due to instability or loss of the R-prime.

8.9 DISCUSSION

Plasmid pMN2 was used in this study to construct R-prime plasmids carrying genomic segments of strain NGR234 DNA involved in exopolysaccharide production. The kanamycin-resistance marker of Tn*5* was used to select for R-primes carrying segments of the NGR234 genome involved in EPS biosynthesis. Both genetic and physical analysis demonstrate that a DNA region containing the Tn*5* insertion site of

mutant ANU2811 has been inserted into the R-prime plasmid. The genetic analysis showed: (a) that the kanamycin resistance of Tn*5* was genetically linked to the tetracycline resistance marker located on the plasmid vector pMN2 and; (b) that transfer of the R-prime plasmids to strain ANU280 or strain ANU265 (a Sym plasmid-cured derivative of NGR234) affected the EPS production of both of these strains. The physical studies indicated; (a) that the sizes of all R-primes were significantly larger than could be expected if Tn*5* simply transposed onto pMN2; (b) all R-primes had a single Tn*5* copy which was present on a restriction fragment of the same size as observed in the original mutant strain; and (c) that the position of the tandem duplication region of *IS*21 was different in the R-prime constructs. The later data suggested that the transposition of IS*21* from the duplication segment is responsible for R-prime formation as has been implied for the formation of other R-primes (*P. aeruginosa* PAO, Leemans *et. al.*, 1980; *M. methylotrophus* ASI, Moore *et. al.*, 1983; *Rhizobium* strain NGR234, Nayudu and Rolfe 1987). Presumably the foreign DNA is inserted between the two copies of *IS*21.

An important observation was that the R-primes were highly stable even in the wild-type strain NGR234 background. This is different from the observation in *R. meliloti* (Banfalvi *et. al.*, 1983) but consistent with the stability of R-primes carrying Sym plasmid genes in strain NGR234 (Nayudu and Rolfe, 1987).

The mutant strain ANU2811 is particularly interesting because the transferred mutant allele is dominant in the parent strain. The presence of R-prime plasmids carrying the mutant allele 2811 not only altered the mucoid colony phenotype of the wild-type strain but also changed its symbiotic properties. There are several important

consequences of this finding. First, this provides further evidence that the EPS produced by the invading *Rhizobium* is essential for the formation of a functional nitrogen-fixing nodule. Secondly, the stability and the dominant phenotype of these Tc^r Km^r R-primes combined with the occurrence of rare Exo^+ "revertants", enabled the isolation of R-primes containing wild-type *Rhizobium* DNA sequences. Genetic and molecular evidence presented is consistent with the formation of these wild-type R-primes being due to a low level of double-reciprocal crossover events which replaced the dominant mutated allele with the corresponding wild-type sequences. The consequence of possessing multiple copies of the wild-type allele on the pMN2 replicon and the mutated allele on the chromosome was the suppression of the dominance effect. Therefore it can be concluded that the dominance effect occurs only when the mutated allele is multicopy with respect to the wild-type allele. The R-prime plasmids containing wild type DNA sequences now make complementation studies possible between the various Exo^- mutants. Finally, it suggests that the altered genetic region of ANU2811 encodes a regulatory element(s). A possible explanation of this induced Exo^- phenotype is that the proposed altered gene product of the 2811 gene interferes with regulation of EPS production in the strain ANU2811. A detailed genetic and physical analysis of the 2811 locus will provide a clearer understanding of the nature of this dominant phenotype.

Interestingly, R-prime plasmid R'2811-9 was able to alter both colony morphology and symbiotic phenotype of mutant strains ANU2861 and ANU2866, but unable to restore their *ade*$^-$ phenotype. These results further support the possibility that the *ade*$^-$ phenotype of both mutants are not responsible for their Nod^- phenotype (sections

3.4 and 3.7). These results also suggest that surface polysaccharide(s) or something related to the polysaccharide synthesis produced by mutant ANU2861 is involved in the induction of a hypersensitive type reaction on siratro by mutant ANU2861 (Figs. 3.4-3.6).

8.10 ACKNOWLEDGMENTS

James X. Gray and Steven P. Djordjevic are thanked for kindly providing probe plasmid DNA. Thanks also to James X. Gray for his cooperation in this chapter.

Table 8.1 Effect of plasmid R′2811-9 on colony morphology of different bacterial strains

Donor strain	Colony phenotype	
	Recipient strain	Transconjugant
HB101(R′2811-9) Km^rTc^r	ANU280, Exo^+, Rif^r	Exo^-, $Rif^rKm^rTc^r$
	1021, Exo^+ Sp^r,	Exo^-, $Sp^rKm^rTc^r$
	USDA191, Exo^+,	Exo^-, Km^rTc^r
	ANU843, Exo^+	Exo^+, Km^rTc^r
	ANU845, Exo^+, Sp^r	Exo^+, $Sp^rKm^rTc^r$
	6015, Exo^+, Rif^r	Exo^+, $Rif^rKm^rTc^r$
	C58, Exo^+	Exo^+, Km^rTc^r
	A136, Exo^+	Exo^+, Km^rTc^r

Exo^+ = mucoid, Exo^- = non-mucoid

Fig. 8.1 Visualization of plasmids in *E.coli* strain HB101 Km^r transconjugants using a modified Eckhardt technique. The tracks show (2) R'2811-1; (3) R'2811-2; (4) R'2811-3; (5) R'2811-4; (6) R'2811-5; (7) R'2811-7; (8) R'2811-8; (9) R'2811-9; (10) R'2811-10; (11) R'2811-12. Track 1 shows the parent plasmid pMN2 visualized in strain HB101.

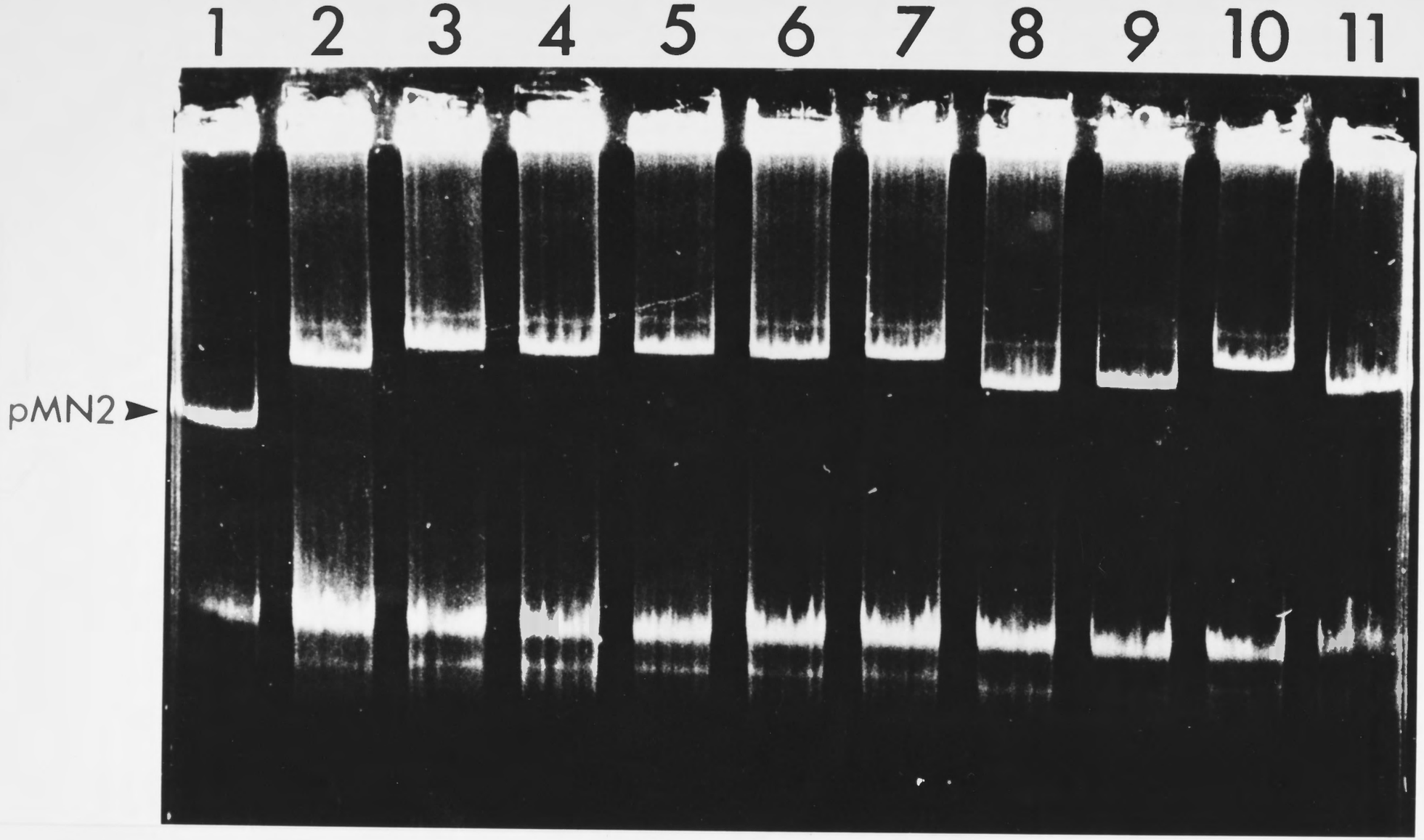

1 2 3 4 5 6 7 8 9 10 11
pMN2

Fig. 8.2 Autoradiograph showing hybridization of a Tn*5*-specific probe to purified R-prime DNA of R'2811-1 (track 2), R'2811-2 (track 4), R'2811-8 (track 5), R'2811-9 (track 6) and ANU2811 total DNA (track 3) digested with *Cla*I. Track 1 contains end labelled λ *Hin*dIII used for size standards. In all cases hybridization was observed to a 8.1 kb *Cla*I fragment.

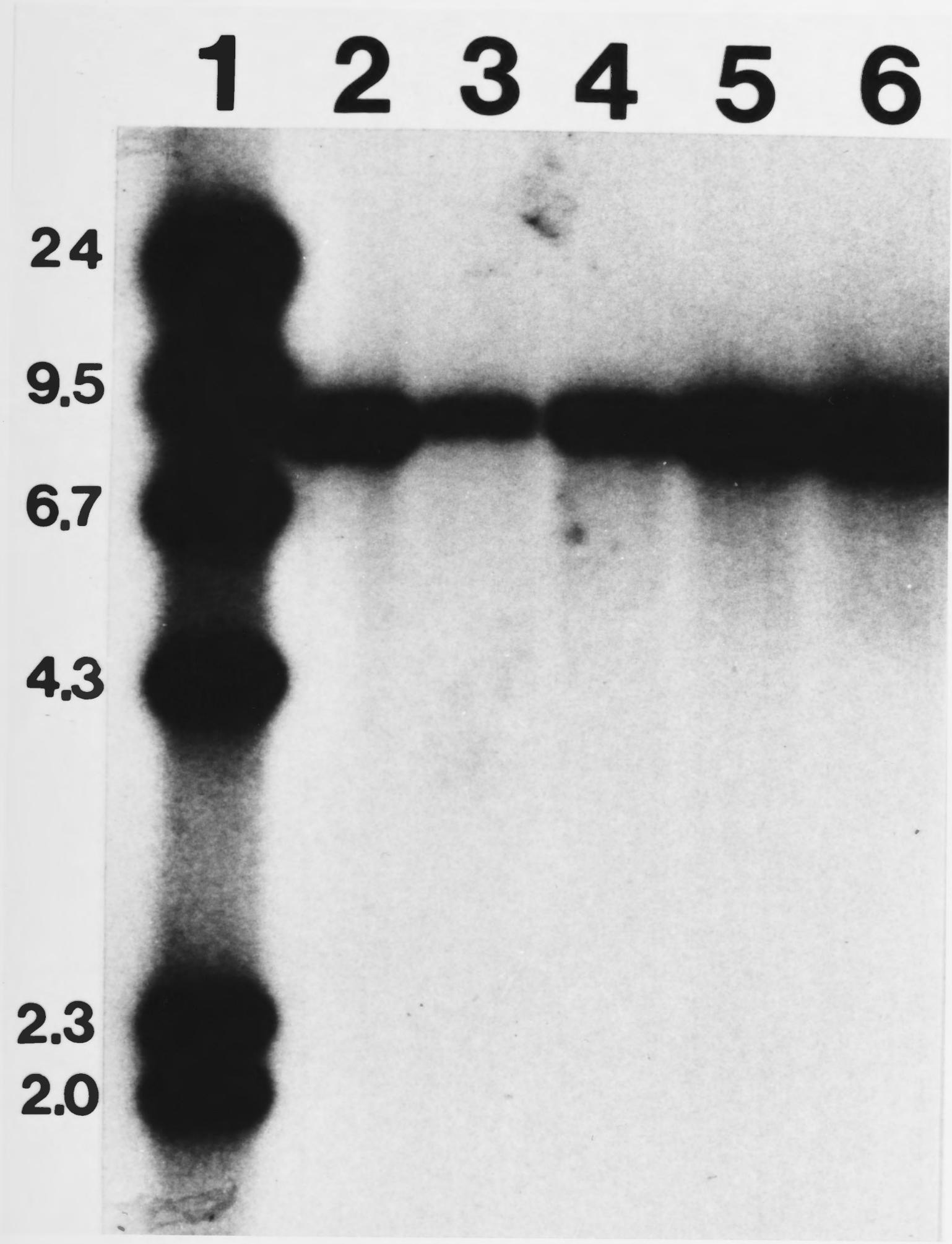

1 2 3 4 5 6
24
9.5
6.7
4.3
2.3
2.0

Fig 8.3 Restriction enzyme digestion of purified plasmid DNA of (2) pMN2; (3) R'2811-1; (4) R'2811-2; (5) R'2811-8; (6) R'2811-9 digested with *Sma*I. Track 1 contains λ *Hin*dIII and *Eco*RI fragments used for size standards.

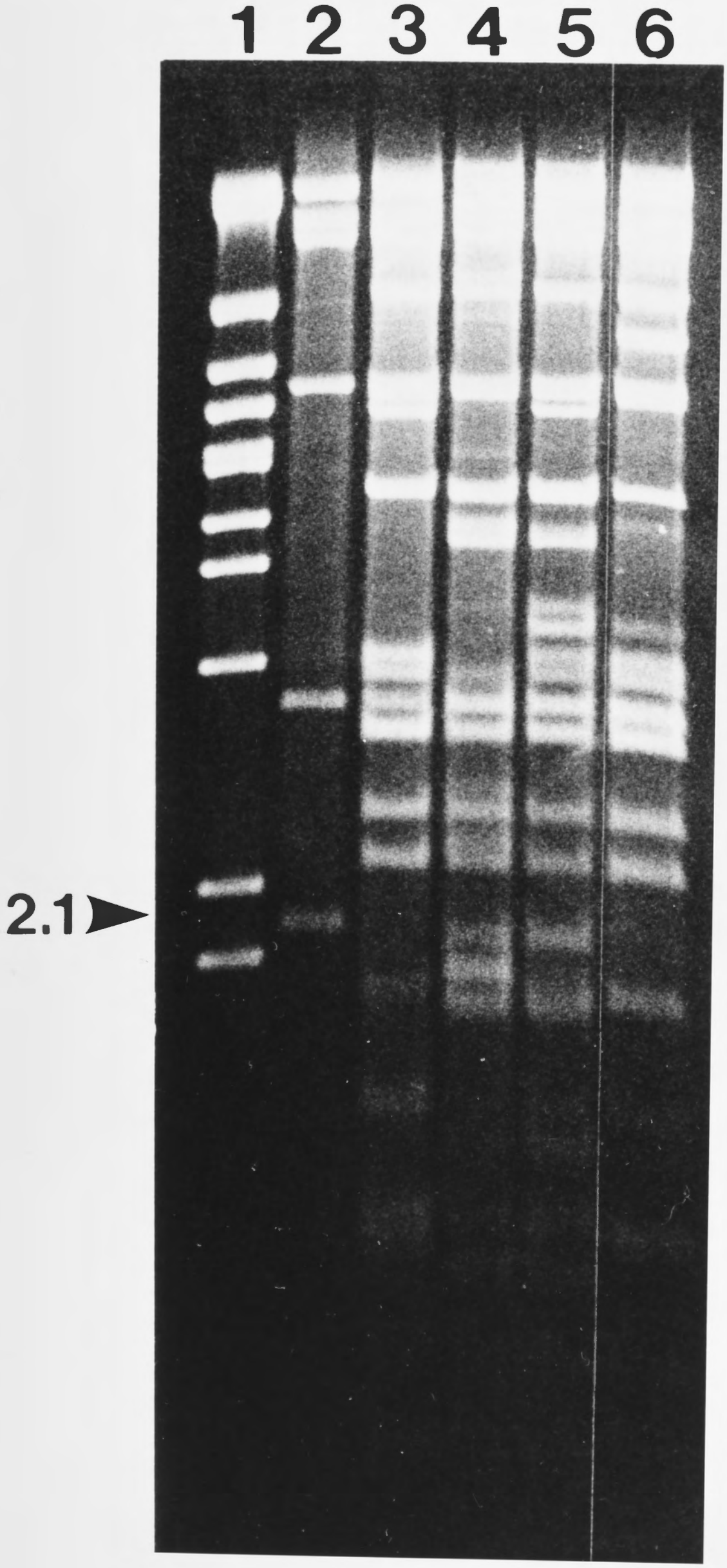

1 2 3 4 5 6
2.1

Fig 8.4 Colony morphologies of strain ANU280 and its derivatives. All strains were grown on selective BMM plates. (a) Mutant ANU2811, rough; (b) ANU280, mucoid; (c) ANU280(R'2811-9), rough.


a
b
c

Fig. 8.5 Siratro nodulation. (a, top left) Wild-type strain ANU280, $Nod^{+}Fix^{+}$; (b, top right) mutant ANU2861, Nod^{-}; (c, bottom left) ANU2861(R'2811-9), $Nod^{+}Fix^{-}$; (d, bottom right) ANU2861(R'3222), Nod^{-}. Nitrogen-fixing activities were measured by acetylene reduction on 4 week old plants

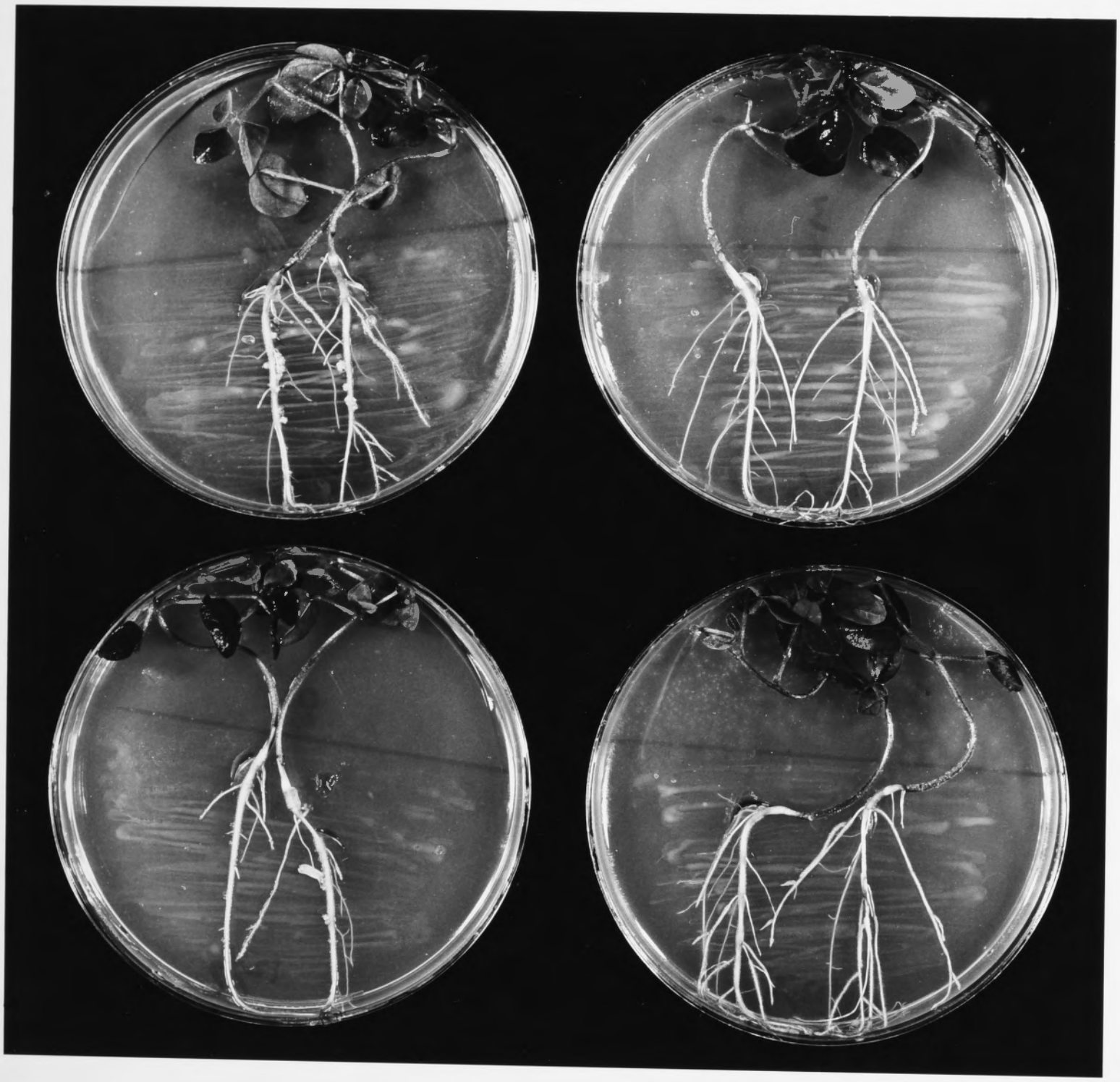


Fig. 8.6 Restriction endonuclease digestion profiles of R-primes. Plasmid DNA was digested with endonuclease *Cla*I. The digested DNA was run in a 0.8% agarose gel. Lanes 1 and 11, *Hind*III digested phage λ marker DNA; lane 2, R'2811-1; lane 3, R'2811-2; lane 4, R'2811-8; lane 5, R'2811-9; lane 6, pMN2; lane 7, R'3205; lane 8, R'3222; lane 9, R'3201; lane, 10, R'3211. The 2.1kb fragment in pMN2 was missing in all R-prime plasmids.

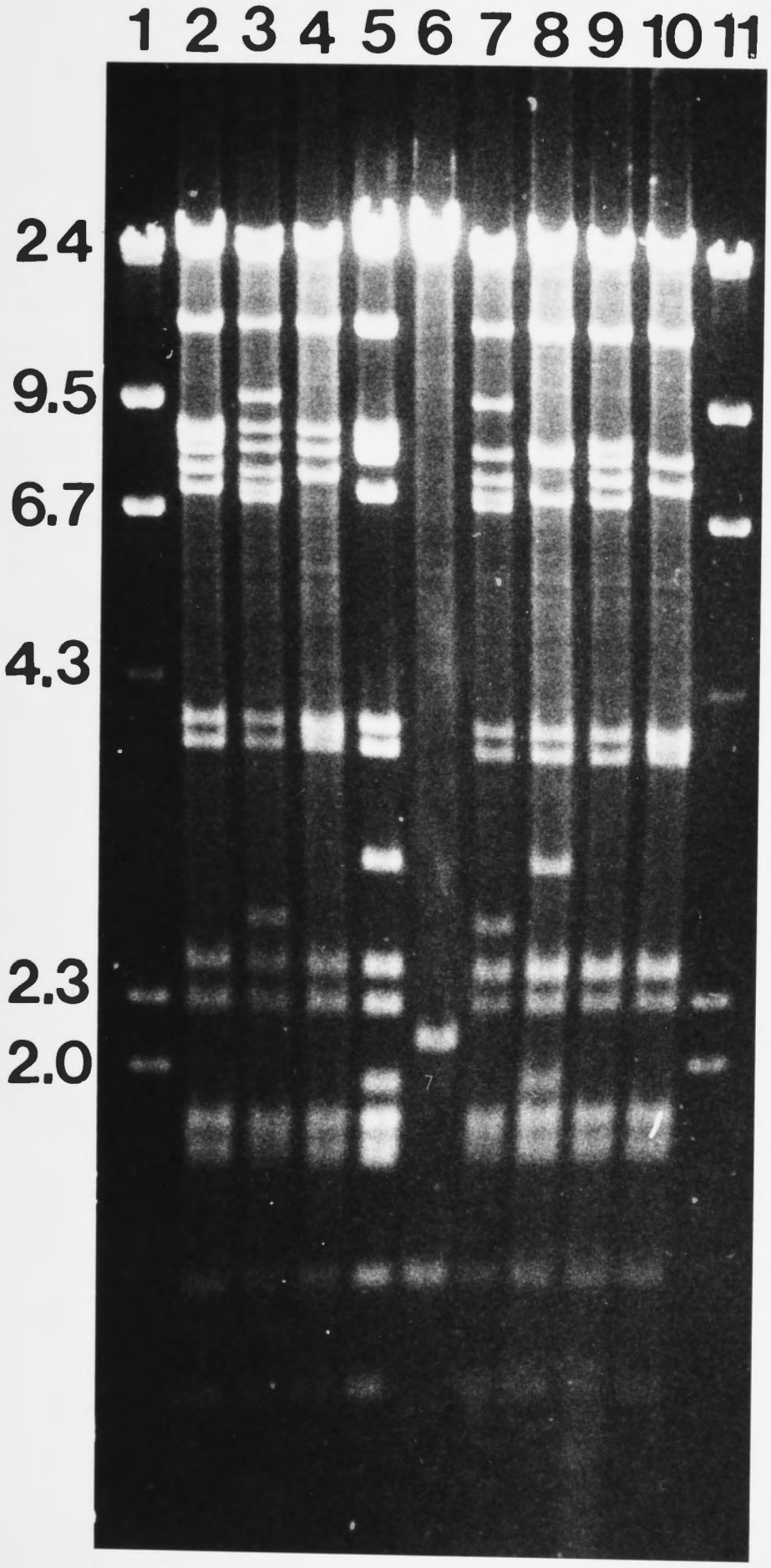
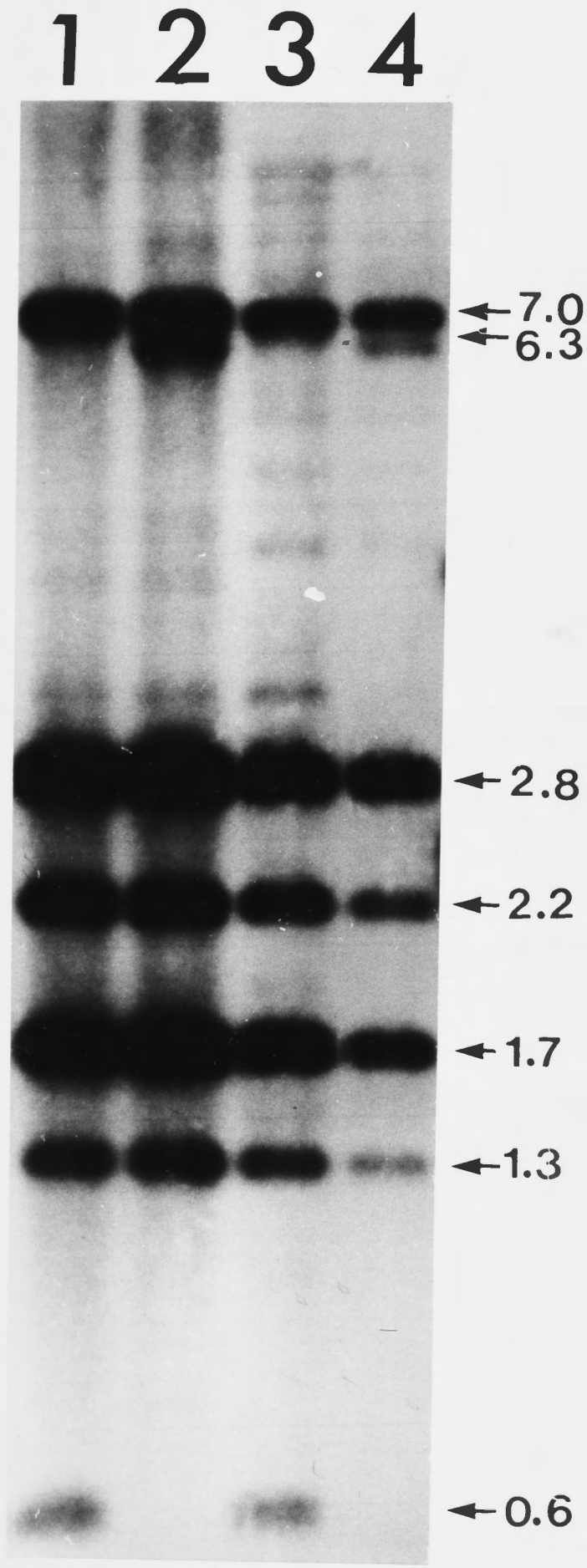

1 2 3 4 5 6 7 8 9 10 11
24
9.5
6.7
4.3
2.3
2.0

Fig. 8.7 Autoradiograph showing hybridization of pJG11 insert DNA (wild-type DNA flanking the 2811-Tn*5* insertion site) to R-prime and genomic DNA digested with restriction endonuclease *Eco*RI. Track 1, plasmid R'3222; track 2, plasmid R'2811-9; track 3, genomic DNA of ANU280 and track 4, genomic DNA of ANU2811. In all cases hybridizing bands are the same except that the 0.6kb fragment present in wild-type DNA sequences is replaced by a 6.3kb fragment in mutant DNA sequences. The experiment was done jointly with James X. Gray.


1 2 3 4
7.0
6.3
2.8
2.2
1.7
1.3
0.6

CHAPTER NINE

GENETIC ANALYSIS OF EXOPOLYSACCHARIDE-DEFICIENT MUTANTS OF *RHIZOBIUM* SP. STRAIN NGR234

9.1 INTRODUCTION

In Chapter Eight, self-transmissible R-prime plasmids carrying genes involved in EPS synthesis were constructed which code for wild-type bacterial DNA sequences of strain NGR234. Despite carrying large segments of the bacterial genome, they were remarkably stable when introduced to derivatives of strain NGR234. In this chapter, another set of R-prime plasmids carrying Tn*5* insertions from different Group 2 Exo^- mutants have been isolated. These R-primes resulted from double reciprocal recombination events between the R-prime carrying wild-type DNA segment, R'3222 (section 8.6), and the corresponding homologous DNA sequences of the Tn*5*-induced Exo^- derivatives of strain NGR234. By using these R-prime plasmids for complementation analysis, several genetically-linked *exo* loci which are involved in EPS synthesis in strain NGR234 have been identified.

9.2 COMPLEMENTATION BY R-PRIME PLASMIDS

Four R-prime plasmids (R'3201, R'3205, R'3211 and R'3222), which carry up to 78kb of wild-type DNA from strain ANU280 (section 8.6), were separately transferred into the ninety polysaccharide-defective mutants of strain ANU280 (Table 3.1). The DNA carried by all four R-primes

spans the Tn*5* insertion site of the Exo$^-$ mutant ANU2811 (section 8.6). The Exo$^-$ colony morphologies for 28 of 30 Group 2 Exo$^-$ mutants were restored by each of the R-prime plasmids (Fig. 9.1, Table 9.3), but mutants from the other eight exopolysaccharide-defective groups were not. This indicates that the majority of the Group 2 mutations are located in the same region of the genome, which happens to be the DNA region in common to all the R-prime molecules (approximately 46kb of overlapping DNA). Since the two mutants, ANU2818 and ANU2831, were not complemented it was concluded that these mutations were located elsewhere on the genome. The presence of intact R-prime plasmids in the ANU2818 and ANU2831 transconjugants was verified by transfer of the plasmids to the Group 2 mutants where, as expected, normal complementation occurred.

None of the Group 2 mutants form normal nitrogen-fixing nodules on the tropical legume *Leucaena leucocephala*, but instead induce callus-like structures which are Fix$^-$ (Table 3.3). Plants inoculated with the Exo$^+$ transconjugants (containing the wild-type R-primes) produced Fix$^+$ nodules typical of those formed by the wild-type strain ANU280 (Fig. 9.2).

9.3 CONSTRUCTION OF R-PRIMES CARRYING TN*5* INSERTION REGIONS FROM DIFFERENT GROUP 2 EXO$^-$ MUTANTS

Because Exo$^-$ mutants carrying the wild-type R-prime plasmids are merodiploid for the *exo* alleles, the mutated regions can be exchanged with homologous wild-type DNA by marker-exchange recombination events. To isolate such R-prime plasmids, R'3222 was introduced into each of the 27 Tn*5*-induced Exo$^-$ mutants from Group 2 that were complemented to Exo$^+$.

One purified Exo$^+$ transconjugant from each mating was then mated with *E.coli* strain HB101 and selected for the transfer of the kanamycin resistance marker of Tn*5*. Kanamycin-resistant (Km^r) colonies arose at a frequency of about 10^{-7} per recipient. Plasmids from Km^r HB101 colonies were able to co-transfer Km^r and Tc^r markers into a Rif^r derivative of HB101 at a frequency of 100%. Analysis of several transconjugants showed the presence of R-prime molecules which were of similar size to original plasmid (R'3222).

To confirm that R-prime plasmids carrying Tn*5* resulted by marker exchange, rather than simply by Tn*5* transposition, plasmid DNA from the Km^r HB101 transconjugants and total DNA isolated from the corresponding *Rhizobium* Exo$^-$ mutants, were digested with *Cla*I and hybridized to Tn*5*-specific sequences (Fig. 9.3). Tn*5* lacks a restriction enzyme site for *Cla*I. Each of the 27 different Km^r R-primes generated, possessed a positive hybridization fragment which co-migrated with the Tn*5*-containing fragment of the original Exo$^-$ mutant (Fig. 9.3). Analysis of restriction enzyme digests showed that, apart from the change in migration of the *Cla*I fragment containing Tn*5*, no deletions or rearrangements could be detected in these Km^r R-primes when their restriction profiles were compared with that of R'3222. This confirms that the Km^r R-primes resulted from double reciprocal recombination events between cloned wild-type sequences in R'3222 and homologous genomic sequences of the *Rhizobium* Exo$^-$ mutants. Through these procedures, the mutated regions of 27 Group 2 mutants were cloned to create a new series of R-prime plasmids (Fig. 9.4).

9.4 GENETIC CLASSIFICATION OF GROUP 2 EXO$^-$ MUTANTS

Since, (a) the introduction of R-prime plasmids carrying the mutated allele of Exo$^-$ mutant ANU2811 was dominant when present in the (normally Exo$^+$) background of ANU280 and (b) this depended upon the elevation of the copy number of the mutated allele, each newly-mutated R-prime plasmid was transferred to ANU280 to determine if any of these plasmids had similar dominant effects on the Exo$^+$ phenotype. Sixteen of 27 ANU280 transconjugants were Exo$^-$. Upon closer observation, two classes of Exo$^-$ transconjugant colonies could be distinguished as 8 of the 16 became partially mucoid after 4 days incubation (Fig. 9.5), whereas the remainder retained an Exo$^-$ phenotype even after 12 days incubation. This phenomenon was still observed after transfer of the R-primes from the cells derived from the semi-mucoid colonies to strain HB101 and back to strain ANU280. This indicated that the semi-mucoid phenotype of these transconjugants was not due to plasmid instability. The Group 2 mutants were divided into three sub-groups on the basis of the expression of their R-primes in strain ANU280 (persistent dominant; leaky dominant and non-dominant) (Table 9.1). Mutants from the two dominant sub-groups were complemented by all 11 R-prime plasmids from the non-dominant group (Table 9.2), suggesting that mutations were in different genes. As with the persistent dominant locus, there might be a requirement for the leaky dominant locus to be elevated in copy number (with respect to the corresponding wild-type allele) for the dominant phenotype to be seen.

Because the introduction of the R-primes carrying mutations in the two dominant loci resulted in Exo$^-$ transconjugants, these could not be used for complementation analysis. However, four distinct

complementation groups were observed when the 11 R-primes from the non-dominant sub-group were transferred into the corresponding non-dominant Exo$^-$ mutants (Table 9.2).

9.5 MOLECULAR ANALYSIS OF R'3222

The mapping studies were done jointly with MR James X. Gray. Using a hybridization probe containing the cloned *Rhizobium* sequences surrounding the Tn*5* insertion site of mutant ANU2811, it was shown that this mutation was located on a 10kb *Bam*HI fragment. This 10kb *Bam*HI fragment was cloned from R-prime plasmid R'3222 into plasmid pUC18 by James X. Gray and a physical map was determined. ^{32}P-labelled internal *Eco*RI fragments were probed onto *Eco*RI digests of total DNA of the *Rhizobium* Group 2 Exo$^-$ mutants. These genomic DNA digests of Exo$^-$ mutants were also probed with radioactively labelled Tn*5* DNA. The Tn*5* insertion sites for 25 Exo$^-$ mutants were mapped on, or very near to, the 10kb *Bam*HI fragment. The Tn*5* insertion sites were localized to 3 *Eco*RI fragments: 0.6kb, 1.3kb and a 7.0kb fragment, which only partly overlapped the 10kb *bam*HI fragment (Fig. 9.6). Based upon physical mapping and complementation data, the Group 2 mutants were further classified into seven distinct genetic groups (A, B, C, D, E, F and G) (Table 9.3).

The Tn*5* insertion sites of genetic groups A, B, and D mapped on the overlapping 7.0kb *Eco*RI fragment. The Tn*5* insertion sites of the two dominant genetic groups mapped to adjacent *Eco*RI fragments located on the 10kb *Bam*HI fragment (Fig. 9.6). All mutations within a 0.6kb fragment and two within an adjacent 1.3kb fragment, were of the persistent dominant genetic group, while the Tn*5* insertion sites for the

leaky dominant genetic group all mapped within the 1.3kb *Eco*RI fragment. The remaining insertion sites of genetic groups C (mutants 2822 and 2824 and G (mutants 2818 and 2831) do not map on, or in, the immediate vicinity of the cloned 10kb *Bam*HI fragment.

Further mapping of Tn*5* insertions involved probing *Eco*RI, *Cla*I and *Xho*II single digests of genomic DNA (from Group 2 mutants) with a specific Tn*5* probe (pKan2). Nine different Tn*5* insertion sites were distinguished (Table 9.4) which, in all cases except one, corresponded with the grouping of the strains into different genetic groups. The exceptional result was that, while mutants in genetic groups A (ANU2820) and B (ANU2826) could be distinguished by complementation analysis (Table 9.2), the site of the insertion of Tn*5* in these two mutants could not be distinguished when genomic digests of these DNA's (using 8 different restriction enzymes) were probed with a Tn*5*-specific probe. Complementation data clearly indicate that the 2820 mutation is *cis* dominant to the mutations at the 2826, and the 2822 and 2824 loci. Mutants 2822 and 2824 (group C) appear to be siblings, as do mutants 2871, 2872, 2873, 2876 and 2877 (group D). The approximate locations of the different mutant groups (Fig. 9.6) clearly show that five to six genes involved in acidic exopolysaccharide synthesis are clustered in a 15kb region of the genome of strain NGR234.

9.6 DISCUSSION

In Chapter Eight, it was observed that Tn*5*-interrupted DNA sequences carried on R-prime plasmids were exchanged with homologous genomic sequences at a frequency of approximately 10^{-7}. Allelic copy number was the factor governing the dominance associated with the 2811-Tn*5* allele

or the corresponding wild-type allele and this permitted the isolation of

R-prime plasmids carrying wild-type genomic DNA sequences spanning the Tn*5* insertion site of strain ANU2811 (a Group 2 Exo$^-$ mutant). In this chapter, it was shown that both colony morphologies and the symbiotic defects for 28 out of 30 Group 2 mutants were corrected by the introduction of these wild-type genomic sequences. This indicates that the mutant loci in the 28 Exo$^-$ mutants are physically clustered to a 46kb region of DNA common to all the R-prime derivatives. Only two Group 2 Exo$^-$ mutants do not map on the DNA carried by the R-prime molecules. Furthermore, 26 of the 28 Exo$^-$ mutants map on, or in the vicinity of, a cloned 10kb *Bam*HI fragment and are clustered over a 15kb region of DNA.

The ability of R-prime DNA to undergo double reciprocal crossover events with homologous genomic sequences was further exploited to allow construction of a set of 28 R-prime plasmids carrying Tn*5* insertions and surrounding genomic DNA sequences. The transposition of Tn*5* from their locations in the *exo* alleles was not detected in any experiment, suggesting that, once in the *Rhizobium* genome, Tn*5* is unable to transpose further. By analysing physical and complementation data at least 5 genes involved in the synthesis of exopolysaccharide in strain NGR234 have been mapped.

Since the Tn*5* insertions in the persistent and leaky dominant alleles (genetic groups E and F) are physically adjacent, it is not clear whether this represents two separate genes or Tn*5* insertions in two separate sites of the same gene. The mutant alleles at loci E and F are dominant to the wild-type alleles only when they are elevated in copy number with respect to the corresponding wild-type allele. When

the positions of the mutant and wild-type alleles are reversed (i.e. the wild-type allele on the R-prime and the mutated allele on the genome), the dominance effect is not apparent. It is concluded, therefore, that a truncated, but bioactive, gene product is still produced after the insertion of Tn*5* into either of these two sites. One possible explanation is that the truncated gene products are fragments of transacting factors which compete for regulatory sites with the wild-type gene products but fail to activate the expression of the target gene or interact with wild-type gene products to yield inactive regulatory products. This would mean that the intracellular concentration of wild-type gene products are also tightly regulated.

There are other reports of mutated alleles being dominant to the corresponding wild-type allele. For example, a mutation in the *capR* gene in *E.coli* affects colanic acid regulation (Markovitz, 1977) and when this mutation is located on an episome it is dominant to the wild-type locus. In this case, mucoid colonies are formed because the *capR* protein is thought to be a repressor protein controlling both colanic acid production and the mucoid phenotype of the resulting colonies.

Complementation data clearly indicate that the mutation at the 2820 locus is *cis* dominant to mutations at two loci (defined by mutations at the 2826 and 2822/24). This is somewhat surprising since (a), physical mapping data were unable to separate the locations of the 2820 and 2826 insertions and (b) the 2822/24 locus is located at least several kb from the 2820 insertion site. Since all these mutations result in a clear Exo^- phenotype, it is concluded that Tn*5* at the 2820 locus has inserted into a key regulatory site (such as a promoter governing the expression of an operon of *exo* genes).

9.7 ACKNOWLEDGEMENTS

James X Gray is thanked for cooperation in the mapping experiment.

Table 9.1. Expression of mutant alleles (carried on R-primes) in the wild-type strain ANU280

R-prime plasmid			Colony morphology of ANU280 transconjugants
R'2807	R'2811	R'2823	Non-mucoid[a]
R'2851	R'2852	R'2854	(persistent)
R'2864	R'2865	R'2890	
R'2808	R'2838	R'2840	Non-mucoid[b]
R'2841	R'2842	R'2844	(leaky)
R'2845	R'2847		
R'2820	R'2822	R'2824	
R'2826	R'2867	R'2871	Mucoid
R'2872	R'2873	R'2875	
R'2876	R'2877		

[a] No visible EPS production of transconjugant after 12 days incubation

[b] Transconjugant was non-mucoid before 4 days incubation but it gradually produced EPS after 4 days continuous incubation and was slightly mucoid in appearance

Table 9.2 Complementaion of Group 2 Exo$^-$ mutants with R-primes from the non-dominant sub-group

Genetic group	Strain	Complementation by R-prime										
		R'2820	R'2826	R'2822	R'2824	R'2867	R'2871	R'2872	R'2873	R'2875	R'2876	R'2877
A	ANU2820	–	–	–	–	+	+	+	+	+	+	+
B	ANU2826	–	–	+	+	+	+	+	+	+	+	+
C	ANU2822	–	+	–	–	+	+	+	+	+	+	+
	ANU2824	–	+	–	–	+	+	+	+	+	+	+
D	ANU2867	+	+	+	+	–	–	–	–	–	–	–
	ANU2871	+	+	+	+	–	–	–	–	–	–	–
	ANU2872	+	+	+	+	–	–	–	–	–	–	–
	ANU2873	+	+	+	+	–	–	–	–	–	–	–
	ANU2875	+	+	+	+	–	–	–	–	–	–	–
	ANU2876	+	+	+	+	–	–	–	–	–	–	–
	ANU2877	+	+	+	+	–	–	–	–	–	–	–
E or F	All members of two dominant sub-groups	+	+	+	+	+	+	+	+	+	+	+

+ Positive complementaion

– No complementation

Table 9.3 Genetic classification of Group 2 Exo$^-$ mutants

Genetic group	Strain			Complementation by R'3222	Phenotype of mutation
A	ANU2820			+	Non-dominant
B	ANU2826			+	Non-dominant
C	ANU2822	ANU2824		+	Non-dominant
D	ANU2867	ANU2871	ANU2872		
	ANU2873	ANU2875	ANU2876	+	Non-dominant
	ANU2877				
E	ANU2808	ANU2838	ANU2840		Leaky
	ANU2841	ANU2842	ANU2844	+	dominant
	ANU2845	ANU2847			
F	ANU2807	ANU2811	ANU2823		Persistent
	ANU2851	ANU2852	ANU2854	+	dominant
	ANU2864	ANU2865	ANU2890		
G	ANU2818	ANU2831		-	Unknown

+ Positive complementation

- No complementation

Table 9.4 Tn*5* location in the Group 2 Exo$^-$ mutants

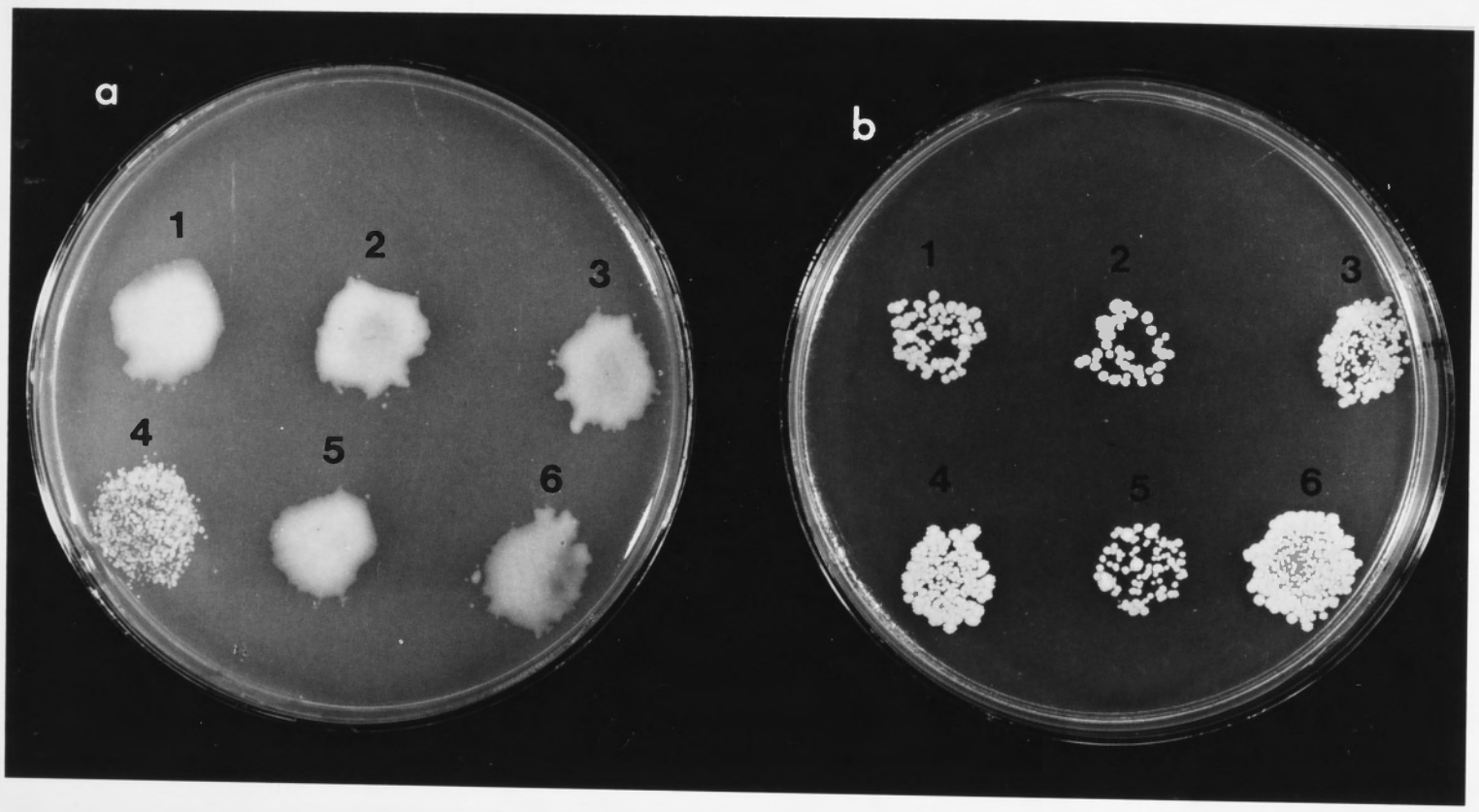
Genetic group	Total DNA isolated from strain		^{32}P-labelled Tn*5* probe hybridized to the DNA fragment(s)* digested with					
			EcoRI	*ClaI*	*XhoI* I			
A	ANU2820		13	6.8	1.3	3.6	2.4	2.5
B	ANU2826		13	6.8	1.3	3.6	2.4	2.5
C	ANU2822	ANU2824	7.5	15	1.2	3.3	2.4	2.5
D	ANU2871	ANU2872						
	ANU2873	ANU2875	13	7.5	0.9	3.6	2.4	2.5
	ANU2876	ANU2877						
	ANU2867		13	7.8	0.7	1.4	2.4	2.5
E	ANU2808	ANU2838						
	ANU2840	ANU2841	7.2	8.1	1.5	3.8	2.4	2.5
	ANU2842	ANU2844						
	ANU2845	ANU2847						
	ANU2823	ANU2890	7.2	8.1	1.7	3.7	2.4	2.5
F	ANU2807	ANU2811						
	ANU2851	ANU2852	6.4	8.1	1.8	3.4	2.4	2.5
	ANU2854	ANU2864						
	ANU2865							
G	ANU2818		10.4	6.4				
	ANU2831		13	6.8	0.9	3.8	2.4	2.5

* The molecular weight of DNA fragment is indicated in kb. Plasmid pKan2 was used as the probe to hybridize to the DNA digested which *EcoRI* or *ClaI*. Plasmid pSUP1011 was used as the probe to hybridize to the DNA digested with *XholI*. Tn*5* has three cleavage sites of *XholI*. The 2.4 and 2.5 Kb *XholI* fragments are the internal Tn*5* sequence.

Fig. 9.1 Colony morphologies of strains ANU2811 and ANU2818 containing R-prime plasmid which carry wild-type DNA sequences of strain NGR234. Donor and recipient strains were mixed on TY plates which were incubated at 29°C overnight and the mating mixtures were then replica-plated onto selective BMM plates. Bacteria on the plates were photographed after 5 days incubation.

(a) 1, ANU2811(R'3201) Exo^+; 2, ANU2811(R'3205) Exo^+;
3, ANU2811(R'3208) Exo^+; 4, ANU2811(pMN2) Exo^-;
5, ANU2811(R'3211) Exo^+; 6, ANU2811(R'3222) Exo^+.

(b) 1, ANU2818(R'3201) Exo^-; 2, ANU2818(R'3205) Exo^-;
3, ANU2818(R'3208) Exo^-; 4, ANU2818(pMN2) Exo^-,
5, ANU2818(R'3211) Exo^-, 6, ANU2818(R'3222) Exo^-.


a
1
2
3
4
5
6
b
1
2
3
4
5
6

Fig, 9.2 Nodulation responses on *Leucaena leucocephala* of ANU280 and its derivatives. (a) Nitrogen-fixing nodules formed by strain ANU280; (b) callus-like structures induced by strain ANU2811 (arrowed); (c) nitrogen-fixing nodules formed by the Exo^{+} transconjugant strain ANU2811(R'3222) and; (d) enlargement of the indicated callus. Nodules photographed 4 weeks after inoculation.


a
b
c


d

Fig. 9.3 Examination of R-prime plasmids carrying mutant alleles of Group 2 Exo$^-$ mutants. Plasmid or total DNA was digested with *Cla*I. The digested DNA was electrophoresed in a 1% agarose gel, Southern blotted and probed with ^{32}P-labelled Tn*5* DNA (pKan2). Lane 1, *Hin*dIII digested λ DNA; lane 2, ANU2807; lane 3, R'2807, lane 4, pMN2, lane 5, R'2824; lane 6, ANU2824.

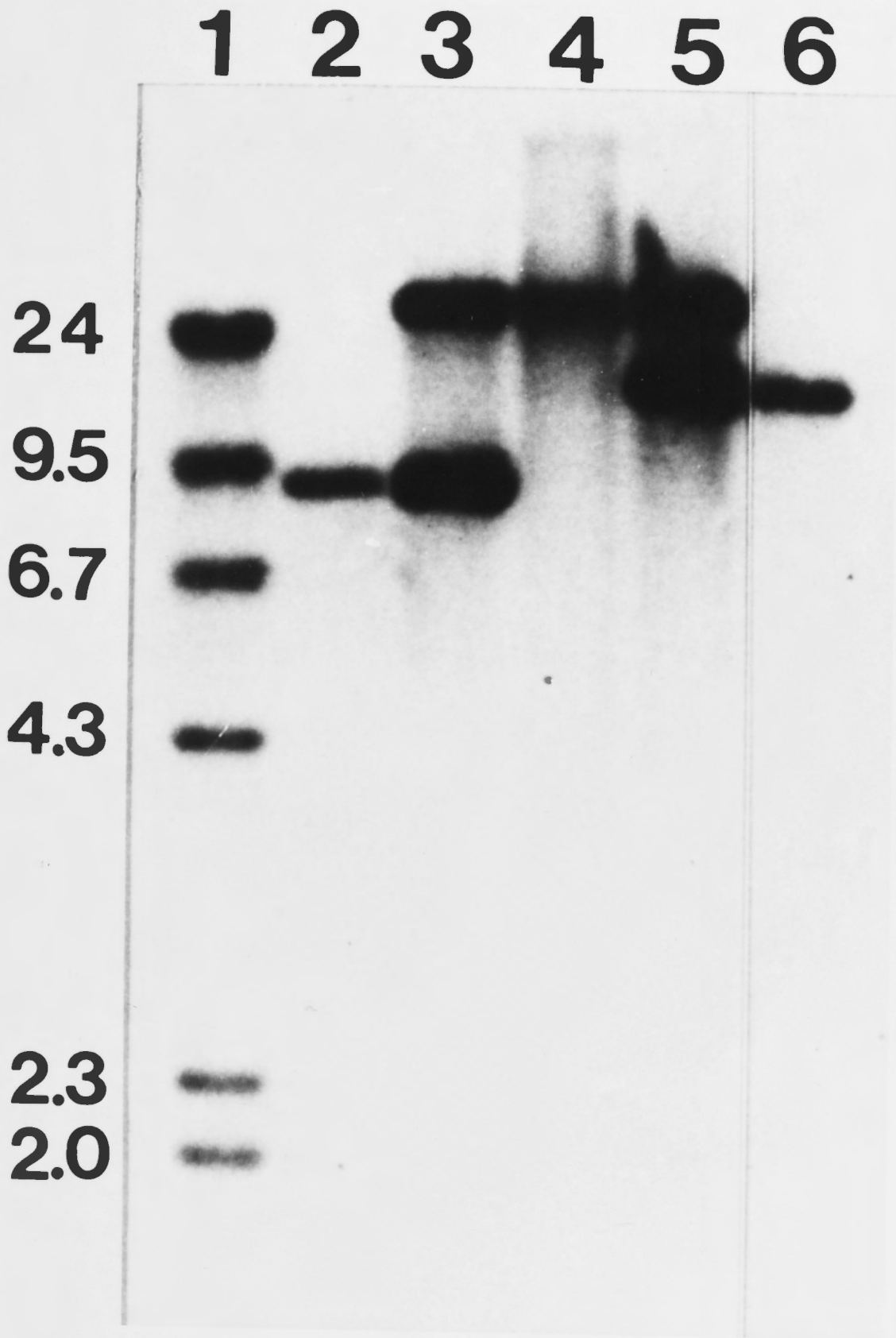

1 2 3 4 5 6
24
9.5
6.7
4.3
2.3
2.0

Fig. 9.4 Construction of the 28 R-prime plasmids carrying Tn*5* induced mutant alleles. Recombination events between wild-type DNA carried on R'3222 and homologous DNA sequences (flanking the Tn*5* insertions) of the mutant strains, resulted in the effective cloning of all mutant alleles onto R-prime plasmids.

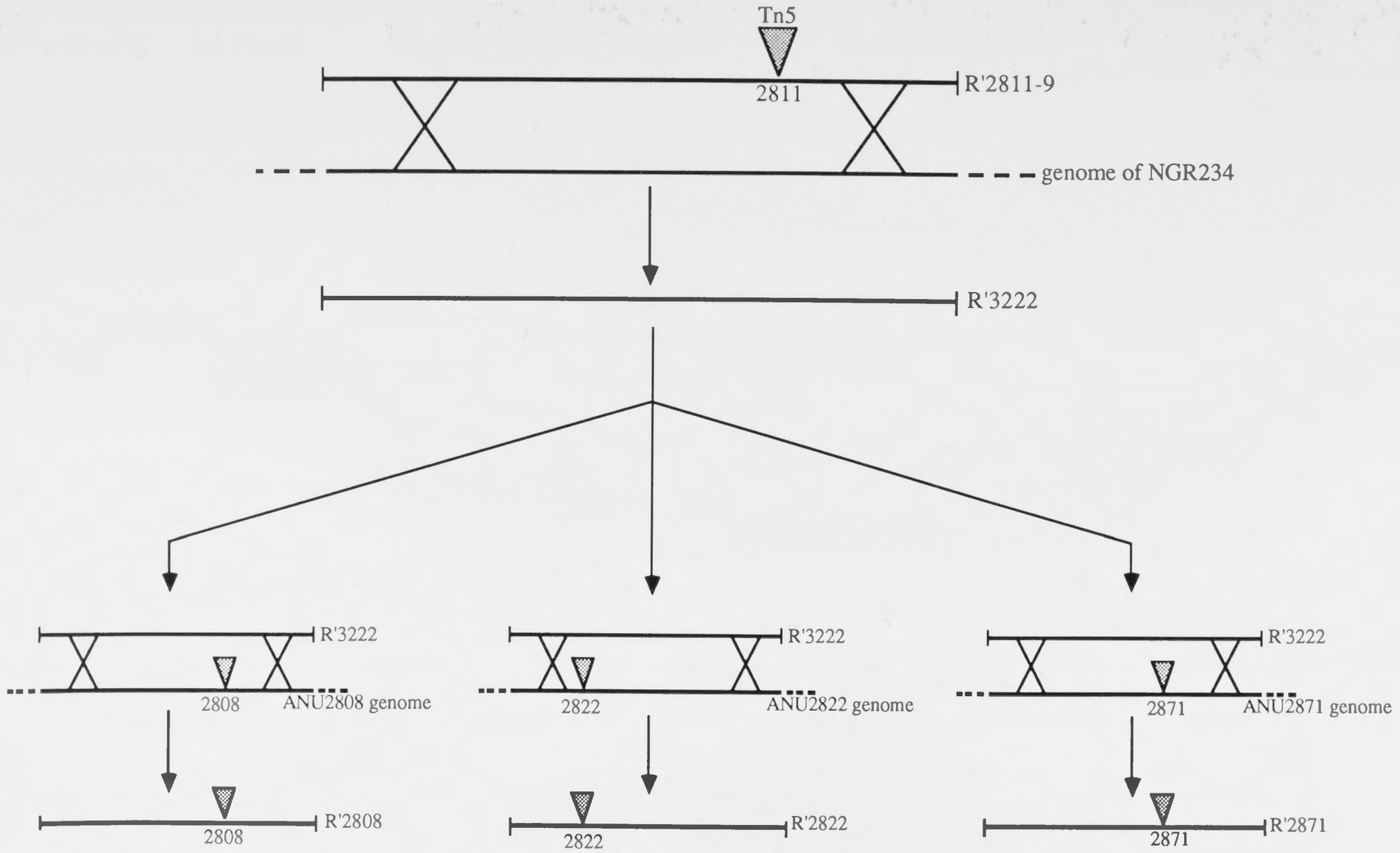

Tn5
2811
R'2811-9
genome of NGR234
R'3222
R'3222
2808
ANU2808 genome
2808
R'2808
R'3222
2822
ANU2822 genome
2822
R'2822
R'3222
2871
ANU2871 genome
2871
R'2871

Fig. 9.5 Demonstration of development of a semi-mucoid colony type in Exo$^-$ mutant strain ANU2841. The Tn*5* insertion in ANU2841 maps in genetic group E (leaky dominant locus). (a) and (b) show the parent ANU280 and mutant ANU2841 strains respectively. (c) and (d) show the colony morphology of ANU280 containing the R-prime derivative with the 2841 Tn*5* insertion after 4 and 6 days incubation respectively. Note the semi-mucoid appearance of the colonies after 6 days.

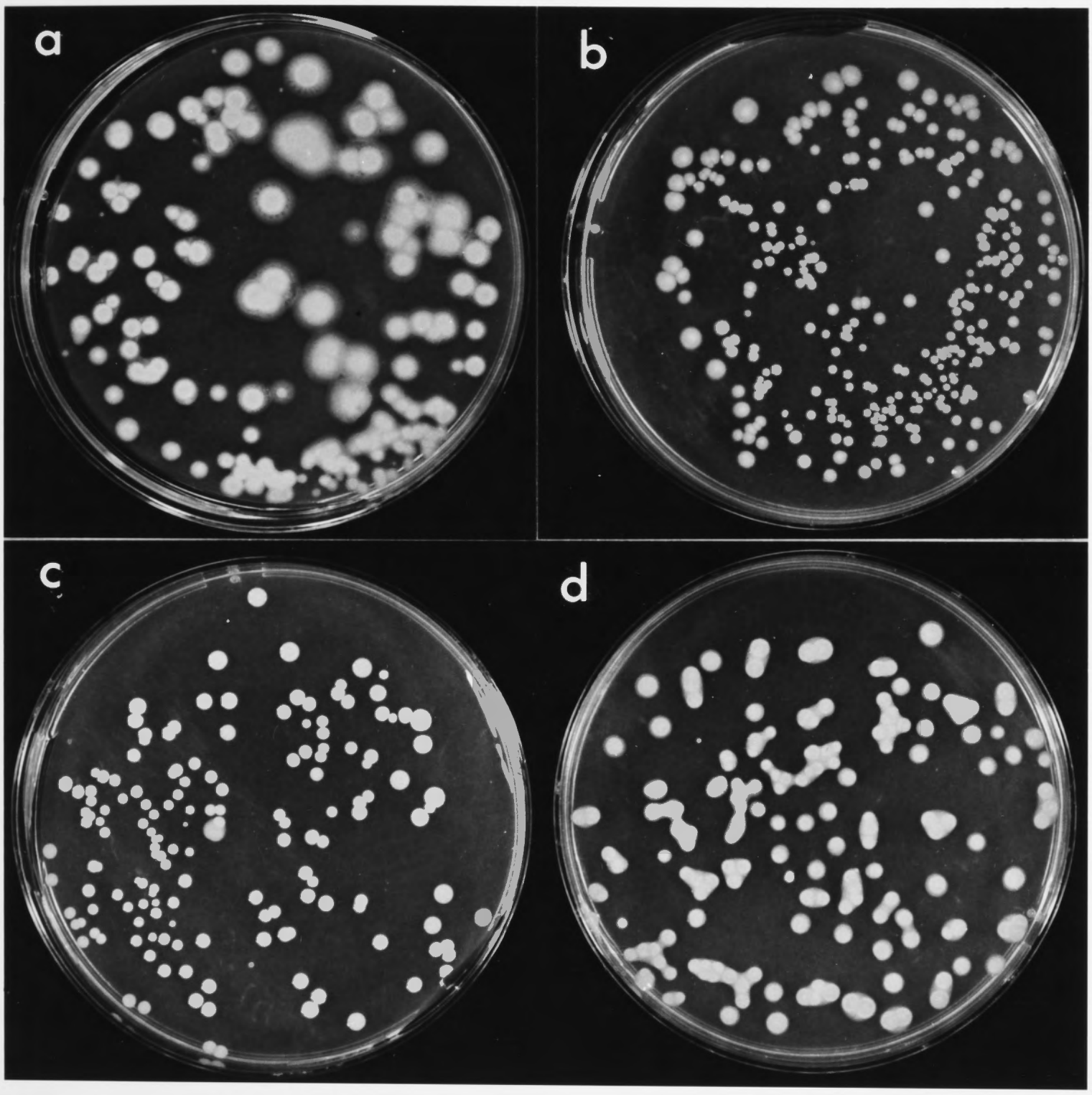
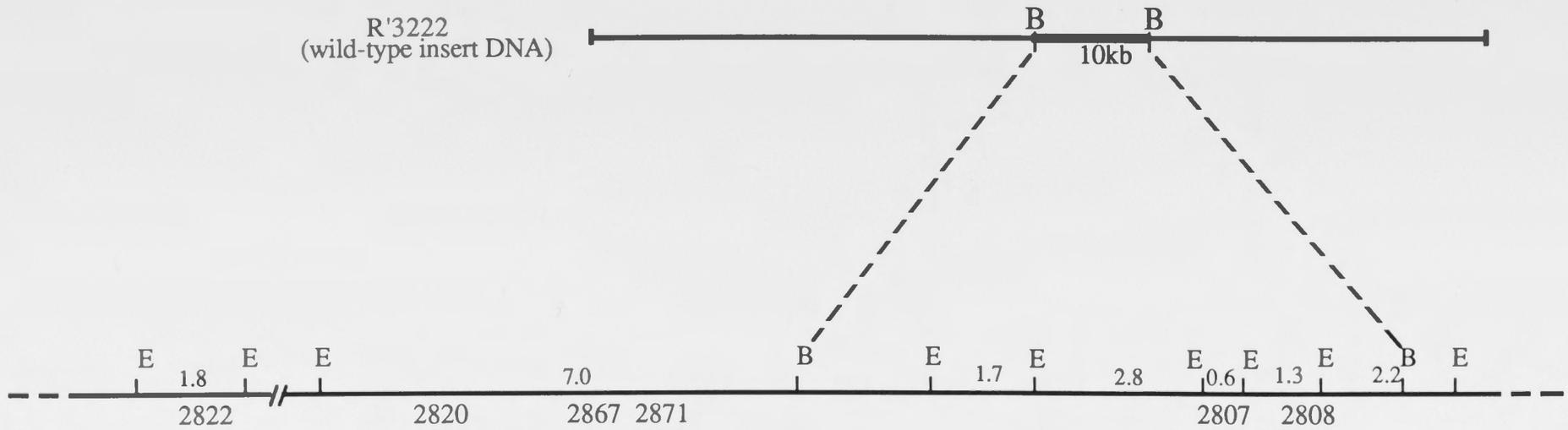

a
b
c
d

Fig. 9.6 Partial restriction map of pJG11 insert DNA, showing approximate Tn*5* insertion sites of Group 2 Exo$^-$ mutants within *Eco*RI fragments. The mutants have been classified into distinct genetic-groups A-F. Distances shown are given in kb and represent distances between *Eco*RI sites only. Restriction sites indicated are: E, *Eco*RI and B, *Bam*HI.

* These mutants belong to genetic-group F.

The experiment was done jointly with James X. Gray.



	2822	2820	2867	2871	2807	2808
	2824	2826		2872	2811	2838
				2873	2851	2840
				2876	2852	2841
				2877	2854	2842
					2864	2844
					2865	2845
						2847
						2823*
						2890*
genetic-groups	C	AB	D		F	E

Table 10.1 Summary of the achievements in this thesis

Chapter	Achievements
Three	Ninety Tn*5* induced mutants defective in EPS synthesis were characterized and classified into nine phenotypic groups. Most of the mutants had a single copy transposon Tn*5* probably inserted into the bacterial chromosome. These mutants had varying symbiotic properties on different host plants. Most of rough (Exo^-) mutants induced abnormal nodule (calli) on an indeterminate nodule forming plant *Leucaena leucocephala* and non-nitrogen-fixing nodules on siratro. There was no simple correlation between EPS production and effective nodulation.
Four	The calli induced by Muc^- mutants had a disorganized nodule structure with many cells containing osmiophilic droplets. Co-inoculation of *Leucaena* with a Muc^- Nod^+ (callus-forming) Fix^- mutant and a Muc^+ Nod^- mutants of strain NGR234 generally resulted in the formation of nitrogen-fixing nodules. The formation of Fix^+ nodules induced by mixed inocula was correlated with the amount of EPS produced by the Muc^+ Nod^- mutant and related to the viability of the Muc^+ Nod^- cells.
Five	Parent strain NGR234 forms Fix^- nodules on non-legume *Parasponia andersonii*. Group 9 mutant ANU2895 was able to induce Fix^+ nodules on *Parasponia* plants and Fix^- nodules on siratro, *Lablab* and *Desmodium* spp.
Six	*Rhizobium* infection of *Leucaena leucocephala* is via the formation of infection threads in the root hairs on lateral roots. EPS or oligosaccharide repeat-unit from strain NGR234 has no effect on *Leucaena* root hair curling or deformation.
Seven	EPS of oligosaccharide repeat-unit is essential for the restoration of a Fix^+ phenotype to several Group 2 Exo^- mutants when inoculated onto *Leucaena* or siratro plants. The biological function of the EPS is related to its structure. The effect of added EPS on *Leucaena* plants can persist at least up to 48 hours.
Eight	R-prime plasmids were isolated carrying mutated or wild-type genes involved in EPS biosynthesis of strain NGR234. The mutant 2811-allele inhibits EPS production of strain NGR234, *R. meliloti* and fast-growing *R. japonicum* strains when it is transferred separately into these different *Rhizobium* species.
Nine	A 46kb region of *Rhizobium* DNA carried by the R-prime plasmids complemented 28 out of 30 Group 2 Exo^- mutants. The complemented Exo^+ transconjugants formed Fix^+ nodules on *Leucaena* plants. Group 2 mutants were sub-divided into seven distinct genetic groups based upon complementation and physical mapping. Six of these seven loci were clustered together on the 46kb insert in the R-prime plasmid. Three of them are involved in a complex regulation of EPS biosynthesis.

CHAPTER TEN

GENERAL DISCUSSION

Some form of interaction of the surface polysaccharides of *Rhizobium* spp. with the host root cell surface undoubtedly plays a role in the establishment of a nitrogen-fixing symbiosis. A combination of genetic, biological and chemical studies should provide answers to the questions of which surface polysaccharides are involved and how they regulate plant biological functions. The strain NGR234 has proven to be an excellent organism for studies into the role of exopolysaccharide (EPS) in *Rhizobium* infection of plants because a diverse group of EPS synthesis mutants can be isolated. Moreover, this strain provides the opportunity to investigate the differences between various host plants to a particular surface-polysaccharide-deficient mutant. The achievements presented in this thesis are summarized in the opposite table.

10.1 GENETIC APPROACH

a. EPS-defective mutants: Mutants of *Rhizobium* spp. defective in exopolysaccharide synthesis have provided excellent genetic material for the investigation of the role of EPS in *Rhizobium*-plant symbiosis (Sanders *et. al.*, 1978; Napoli and Albersheim, 1980; Law *et. al.*, 1982). Studies with transposon-induced exopolysaccharide-deficient (Exo^-) mutants of *R. trifolii* (Chakravorty *et. al.*, 1982), *R. meliloti* (Leigh *et. al.*, 1985) and *R. phaseoli* (Borthakur *et. al.*, 1986) have

demonstrated that *Rhizobium* genes involved in EPS synthesis are necessary for the establishment of a nitrogen-fixing symbiosis, although these studies have been unable to distinguish whether EPS has a direct or an indirect role in infection.

A central theme of this thesis is the isolation and characterization of 90 Tn*5*-induced mutants in strain NGR234 with altered EPS synthesis. These mutants were classified into 9 different phenotypic groups. The advantages of using these specific mutants to investigate the role of EPS on *Rhizobium* infection are: (a) the defect is due to a single mutation; and (b) the response of different plants to these mutants can be assessed. These mutants are not only defective in their polysaccharide synthesis but also have altered symbiotic properties. Apparently, the alteration of EPS synthesis is effective at different stages of nodule development. The changes of symbiotic properties of the mutants can be subtle on some plants and quite marked on the others.

Biochemical synthesis of bacterial exopolysaccharides is a very complex system consisting of several pathways, such as substrate uptake, intermediary metabolism and polymer synthesis. Each pathway consists of several steps (Sutherland, 1979) and thus mutation at any step of these pathways can alter EPS synthesis. Various mutants of strain NGR234 with altered EPS production appear to be mutated at different steps of the EPS biosynthesis pathway. Many of the mutants of the largest group (Group 2) fail to synthesize acidic EPS which results in the formation of a dry, non-mucoid (Exo^-) colony morphology on various test media and have significant symbiotic defects on *Leucaena leucocephala*. The Exo^- colony phenotype of the Group 2 mutants provides a visible marker for the study of the genetic organization of the *exo* loci in strain NGR234.

b. Genetic organization of *exo* genes: The method developed in this thesis for genetic analysis of Exo^- mutants of strain NGR234 was the construction of different types of R-prime plasmids carrying *Rhizobium* genes involved in EPS biosynthesis. The use of these different types of R-prime plasmids enables precise genetic mapping of the different Group 2 Exo^- mutants and the construction of partial diploids for the study of the control of *Rhizobium* polysaccharide synthesis.

Group 2 Exo^- mutants consist of at least 7 different loci (A, B, C, D, E, F and G). Loci B, C and D may be structural genes encoding enzymes involved in EPS synthesis. The locus A may be a promoter site(s) responsible for divergent transcription of loci B and C since the mutation at the locus A is *cis*-dominant to the mutations at the loci B and C. Divergent transcription for a biosynthetic locus has been observed in the biotin operon in *E.coli* K-12 (Cleary *et. al.*, 1972; Guha *et. al.*, 1971). The biotin operon consists of five structural genes (*bio*A, B, C, D and F) which are clustered and form two transcription units. Both transcripts originate at, and are controlled by, sites between *bio*A and *bio*B (Cleary *et. al.*, 1972; Guha *et. al.*, 1971). Genetic and DNA sequence studies have shown that the *bio*A and *bio*B promoters are not independent (Ketner and Campbell, 1975) and are two partially overlapping face-to-face promoters (Otsuka and Abelson, 1978). Mutations in the overlapping region prevented transcription in both directions (Ketner and Campbell, 1975).

c. Regulation control of *exo* genes: Locus E or F or both may encode a trans-acting positive regulator of EPS synthesis since transfer of the mutation at locus E or F into strain NGR234 inhibits

EPS production. Two positive regulatory genes (*rcs*A and *rcs*b) have been found to control CPS synthesis of *E.coli* K-12 (Gottesman *et. al.*, 1985). Transfer of the mutation at *rcs*A or *rcs*B into mucoid (CPS^+) strains and strains carrying different *cps::lac* fusions completely abolish capsule synthesis and decrease expression of *cps::lac* fusions, indicating that *rcs*A and *rcs*B are positive regulatory loci for *cps* (Gottesman *et. al.*, 1985). Loci E and F may encode a multimeric regulatory complex gene product since the dominance expression of the mutated gene is related to the gene copy number. Transfer of the mutation at locus E or F into fast-growing *R. japonicum* and *R. meliloti* also inhibits EPS synthesis of these two heterologous strains. Moreover, a probe containing E and F loci shows strong hybridization to DNA from these two different *Rhizobium* strains (Gray, personal communication). This suggests that these three different *Rhizobium* strains may share a similar regulation system of controlling polysaccharide synthesis. It has been shown that the symbiotic host-range of strain NGR234 and the fast-growing *R. japonicum* strains overlap considerably and the two strains also have certain similarity in growth on different media (Morrison, 1984).

In *E.coli* K-12, mutations at negative regulatory genes of CPS synthesis cause constitutive synthesis of CPS (Markovitz, 1977). The group 9 mutants over-produce EPS on all test media suggesting that these mutants may carry defects in a negative regulator of EPS production. Interestingly, a gene on the Sym plasmid of *R. phaseoli* when cloned onto a high copy number vector inhibits EPS synthesis (*psi*) and affects nodulation (Borthakur *et. al.*, 1985). Normally, *psi* does not express on the Sym plasmid and its transcription is inhibited by another gene [polysaccharide restoration (*psr*)] on the Sym plasmid

(Borthakur and Johnston, 1987). DNA sequence analysis indicated that the *psi* specified a small polypeptide which was thought to be associated with the membrane of *Rhizobium* and prevented the transport or processing of molecules required for EPS production (Borthakur *et. al.*, 1987).

It has been suggested that *Rhizobium* EPS production is altered by the switchover from the free-living to bacteroid state (Bassett *et.* al., 1977a; Goodchild and Bergersen, 1966; Jordan *et. al.*, 1963; Newcomb, 1976; Newcomb and McIntyre, 1981; Patel and Yang, 1981). Infection threads of some legume-*Rhizobium* symbiosis contain an electron-dense "thread matrix", inferred to be EPS secreted by the invading rhizobia (Jordan *et. al.*, 1963; Newcomb, 1976; Newcomb and McIntyre, 1981; Patel and Yang, 1981). Bacteria emerging from the ends of the infection threads may carry some of this matrix with them (Newcomb, 1976; Newcomb and McIntyre, 1981). However, little or none of this material is seen to surround mature bacteroids in alfalfa (Jordan *et. al.*, 1963) and soybean (Goodchild and Bergersen, 1966), and the vesicles which formerly contained it may be degraded by the host cells after the blebbing-off of the bacteroids (Bassett *et. al.*, 1977a; Newcomb and McIntyre, 1981). Chemical analysis has shown that only low levels of *Rhizobium* EPS are present in mature soybean nodules (Tully and Terry, 1985). van Brussel *et. al.* (1977) have also described that bacteroid walls have lower carbohydrate and heptose (a constituent of lipopolysaccharide) contents than bacterial walls.

These observations suggest that *Rhizobium* have a complex regulation system consisting of a number of positive and negative regulatory genes to control EPS synthesis in both free-living and symbiotic states. In the free-living state, a positive regulatory

system must be dominant to a negative regulatory system so that bacteria produce EPS and form mucoid colonies. In the symbiotic state, however, the negative regulatory system may be dominant to the positive regulatory system that decreases EPS production. In the symbiotic state, EPS synthesis may be controlled by bacterial factors or plant factors or by the interaction between the plant and bacteria. Several studies have shown that free O_2 concentration is one factor that regulates EPS synthesis by *Rhizobium* (Agarwal and Keister, 1983; Pankhurst and Craig, 1978). The low free O_2 in a nodule may also inhibit bacterial synthesis of EPS (Tully and Terry, 1985). Because EPS synthesis requires a large amount of energy supply (Markovitz, 1977; Jarman and Pace, 1984), decrease of EPS production in the symbiotic state, particularly after bacterial release into cytoplasm, may increase the available ATP synthesis for nitrogenase to fix nitrogen. It is also possible that the release of bacterial EPS from the bacteroid state may elicit the plant defence response.

10.2 EVALUATION OF EXOPOLYSACCHARIDE IN INFECTION

a. Host-specific nitrogen fixation: The majority of the EPS defective mutants of strain NGR234 fail to induce nitrogen-fixing nodules on one or more host plants and thus become narrower host-range Nod^+Fix^+ *Rhizobium* strains. For example, in the case of mutant ANU2895 the consequence(s) of the mutation enables the strain to form nitrogen-fixing nodules on the non-legume *Parasponia* (Table 5.1) but non-nitrogen-fixing nodules on legumes (Table 3.2). This suggests that normal EPS synthesis of strain NGR234 is involved in host-specific nitrogen fixation. Mutants defective in host specific

fixation (*Hsf*) have been isolated recently in a *Bradyrhizobium* strain (Wilson *et. al.*, 1987). It is possible that host-specific fixation mutants may point to as yet unidentified regulatory steps which exist between the initial induction of a root nodule, and the final establishment of a fully effective nitrogen-fixing symbiosis. A number of *Rhizobium* genes controlling host specific nodulation (*Hsn*) have been characterized recently (Djordjevic *et. al.*, 1985; Downie *et. al.*, 1983; Hombrecher *et. al.*, 1984; Horvath *et. al.*, 1986)). These studies suggest that recognition of a compatible partner between rhizobia and host is not a single step in the interaction but rather the culmination of a series of events.

b. Cellular complementation: Many Exo^- mutants can normally infect *Leucaena* plants when co-inoculated with Nod^- mutants which have normal Exo^+ colony morphologies (e.g. Sym plasmid-cured strain) (Table 4.1). Nitrogen-fixing nodules are often produced in these experiments. A similar observation has been reported in *R. meliloti* (Klein *et. al.*, 1987). Exo^- mutants of *R. meliloti* induce Fix^- nodules without infection threads and intracellularly located bacteria on alfalfa (Leigh *et. al.*, 1985). When an Exo^- mutant was mixed with an Exo^+ Nod^- Fix^- mutant and co-inoculated onto alfalfa then nitrogen-fixing nodules were produced. Interestingly, the cellular complementation did not occur between an Exo^+ Nod^+ Fix^- and Exo^- mutants. It was suggested that, at the molecular level, exopolysaccharides can be modified in some way by nodulation (*nod*) genes (Klein *et. al.*, 1987). The co-inoculation experiments are very important because they provide: (a) evidence suggesting that EPS has a direct role in symbiosis; and (b) which Exo^- mutants might be suitable

for testing the possibility that added EPS does have a direct role during infection of the plant by rhizobia.

c. Direct, active role of EPS in infection: The exogenous addition of specific oligosaccharide or EPS to plants together with Exo^- mutants results in the correction of the inability of these mutants to induce nitrogen-fixing nodules. This result provides the most compelling evidence for a direct role for EPS in the establishment of an effective symbiosis.

Several experiments suggested that the structure of EPS was important for the correction phenomena. The Group 8 mutant ANU2858 produces 10% of wild-type level EPS (Table 3.4). Chemical analysis has shown that the difference between the EPS from the mutant and the EPS from the parent is that the EPS from the mutant does not contain the galactose and glucose residues but had rhamnose residue (Djordjevic S. personal communication). This mutant has a Nod^+ Fix^- phenotype on five test plants. Consequently, the EPS from the mutant is unable to restore the nitrogen fixing ability of Exo^- mutants. EPS isolated from *R. trifolii* strain ANU843 has a structure different from that of strain NGR234 (Carlson *et. al.*, 1986; Djordjevic S. *et. al.*, 1987a). The addition of EPS isolated from strain ANU843 to together with Exo^- mutants of strain NGR234 was unable to facilitate the induction of nitrogen-fixing nodules on *Leucaena* plants (Djordjevic S. *et. al.*, 1987b). Similarly, an Exo^- mutant of *R. trifolii* was able to induce nitrogen-fixing nodules on white clover plants by the addition of the EPS from strain ANU843 but it failed to do so by the addition of the EPS from strain NGR234 (Djodjevic S. *et. al.*, 1987a). EPS from mutant ANU2858 and *R. trifolii* strain ANU843 when adding to Exo^-

mutants induced small, normal-looking, ineffective nodules rather than calli on *Leucaena leucocephala* (section 7.4; Djordjevic S. *et. al.*, 1987b). This suggests that the effect of EPS on *Leucaena* occurs at both an early and later stage in nodule development. For bacteria to pass the early nodule development stage may not require specific EPS but they may require homologous or specific EPS to pass the later nodule development stage. Therefore, this proposed that the stage of later nodule development appears to be a crucial step determining host specific nitrogen fixation.

In *R. meliloti*, Tn*5*-induced mutants of group ExoH induced round white nodules which were unable to fix nitrogen. Although ExoH mutants produced normal amounts of EPS, proton NMR spectroscopy showed that the molecules lacked succinate substitutions (Leigh and Reed, 1987). A similar group of Tn*5*-induced mutants of *R. meliloti* strain 2011 produce three times as much EPS as parent strain and show a nodulation defective phenotype on alfalfa. ^{13}C-NMR analysis of the EPS showed that the polymer produced, lacked pyruvate (Puhler *et. al.*, 1986). These results indicate that the biological function of EPS is related to its structure and minor structural changes in acidic EPS structure can alter the symbiotic proficiency. Thus, the structurally-dependent activity of EPS indicates that it has more than a simple passive role of masking determinants on the *Rhizobium* surface.

The evidence supporting this possibility came from the "washing off" EPS experiments. The purified EPS from strain NGR234 was added to *Leucaena* roots 24 or 48 hours prior to inoculation and then washed off. *Leucaena* plants pretreated with the EPS for 48 hours before washing and inoculated with Exo$^-$ mutants produced nitrogen-fixing

nodules (Table 7.3). This indicated that the effect of EPS on the plant could persist after its potential removal. It is possible that the interaction of EPS with target root hair cells or epidermal cells in the infection zone induces a plant response and once the response is induced it is irreversible. Such a response may lead to a "switch off" of a plant defence system and to create microenvironments in the plant suitable for bacterial invasion. It has been reported that infectivities of *Rhizobium* strains can be enhanced by a pretreatment of soybean root with purified EPS of *R. japonicum* (Bauer *et. al.*, 1979) or after the addition of crude EPS preparations from highly infective *R. meliloti* to a poorly infective *R. meliloti* strain (Olivares *et. al.*, 1984). It was suggested that EPS is capable of inducing in emerging root hairs a state of infectibililty (Bauer *et. al.*, 1979).

Plant-derived polysaccharide-degrading enzymes have been reported to be present in the root exudate of several legumes (Dazzo *et. al.*, 1982; Bhagwat and Thomas, 1984; Solheim and Fjellheim, 1984). *Rhizobium* capsular polysaccharides (CPS) were altered in structure when incubated in the presence of host root exudate. The host induced modifications of *Rhizobium* CPS (HI-CPS) enhanced the nodulation efficiency of wild-type rhizobia when inoculated under low inoculum density (Bhagwat and Thomas, 1984). HI-CPS also provoked rapid root hair curling in cowpea seedlings. Significant curling was observed within 12 to 16 hours after the addition of HI-CPS to the infectible region just above the root tip (non-root hair (NRH) zone). Cowpea root epidermal cells only in the NRH zone synthesized 5-7 new polypeptides specifically in response to HI-CPS or symbiotic *Rhizobium* sp. strain 1001. The characterised peptide bands did not appear in

cowpea root zones inoculated with *R. meliloti* or *E.coli* strain carrying 8.5kb cloned nodulation (*nod*) genes from *R. meliloti*, suggesting that the *de novo* synthesis of plant proteins is induced specifically in response to the added HI-CPS (Bhagwat and Thomas, 1987). Again these results indicate that *Rhizobium* EPS may have a direct, active role in inducing plant responses. Presumably, target root epidermal cells in the infection zone, such as in the NRH zone, can recognize *Rhizobium* surface polysaccharides as signal molecules and in response "trigger off" the plant defence systems

10.3 INDUCTION OF PLANT DEFENCE SYSTEMS

To successfully invade and induce a nitrogen-fixing symbiosis, *Rhizobium* must avoid eliciting a plant defence response, particularly, the hypersensitive reaction (Dickinson and Lucas, 1982). Mutants defective in the synthesis of different surface polysaccharides appear to elicit a plant defence response at different stages of nodule development. Group 9 mutant ANU2861 (Chen *et. al.*, 1985; Ridge, 1985) evokes an early response on siratro. The early infection events (root hair curling and nodule induction) are initiated on siratro plants but infection fails to proceed from this point onwards. This results in the failure to form infection threads and nodule meristems. Group 2 Exo^- mutants (section 4.2) elicit a slightly later response on *Leucaena*. *Leucaena* plants produce abnormal nodules with or without infection threads, vascular bundles and meristematic activities in response to the Exo^- mutants. The other EPS synthesis mutants of strain NGR234 (Table 3.3) apparently elicit a later response on siratro, *Desmodium* spp., *Lablab* or *Leucaena*. These mutants induced

small or large normal-looking but ineffective nodules on the plants. The infection of white clover or alfalfa plants by Exo$^-$ mutants of *R. trifolii* (Chakravorty *et. al.*, 1982) or *R. meliloti* (Leigh *et. al.*, 1985) are blocked apparently at different stages of nodule development. An Exo$^-$ mutant of *R. trifolii* induced root hair curling and small ineffective nodules on white clover plants. Microscopic studies show a delayed nodule development and bacterial release in the infected nodules and most of the bacteria are not enclosed in plant membranes (Chakravorty *et. al.*, 1982). Exo$^-$ mutants of *R. meliloti* which were unable to induce root hair curling on alfalfa plants formed ineffective nodules without infection threads and intercellular bacteria (Leigh *et. al.*, 1985). *R. trifolii* CPS or EPS oligosaccharide fragments enhance the number of infection threads and nodules formed on white clover (Abe *et. al.*, 1984). These observations suggest that *Rhizobium* cell surface polysaccharides may function as signal molecules which induce host responses for the formation of infection threads and thereby enable the bacteria to avoid eliciting plant defence systems.

Tn*5*-induced Exo$^-$ mutants which fail to synthesis acidic EPS induce the formation of small calli on *Leucaena* and small ineffective nodules on siratro. Microscopic analysis of these calli reveals the presence of osmiophilic inclusions in the callus tissue which are absent when the wild-type strain infects (Chen *et. al.*, 1985; Chen and Rolfe, 1987; Djordjevic S. *et. al.*, 1987a). Osmiophilic inclusions produced during incompatible infection by mutant *Rhizobium* are possibly phenolic compounds of phytoalexins which are antimicrobial compounds commonly induced in pathogenic plant interactions. Therefore, the events in the infection of *Leucaena* induced by Exo$^-$ mutants can be

likened to a plant resistance response. The elicitation of a phytopathogenic-like deposition of substances by the plant in a response to infection by Exo$^-$ *Rhizobium* strains suggests that *Rhizobium* is normally able to overcome or avoid stimulation of such a plant defence response by means of its production of exopolysaccharides.

Clearly, symbiotic defects of Exo$^-$ mutants are related to their impaired exopolysaccharide synthesis. Previous examinations of various Exo$^-$ mutant from different fast-growing *Rhizobium* species (*R. trifolii*, *R. leguminosarum* and *R.meliloti*) has shown that they can form small, non-nitrogen-fixing (Nod$^+$Fix$^-$) nodules on their host plants. A general summary of the microscopic studies shows that, while some bacteroid material may be formed, this is readily broken down and the bacteria are usually not released from their infection threads (Bar$^-$ phenotype). There is less meristematic cell growth induced and less ramification of the invading infection thread. These studies were conducted on plants that form indeterminate type nodules (clovers, peas and alfalfa) and the general findings are summarized in Figure 10.1. The results of this thesis also show that *Leucaena*, an indeterminate nodule-forming plant, reacts to Exo$^-$ mutants by forming very defective callus-like-nodule structures on its roots. It should be noted that generally, plants that form determinate type nodules (siratro, *Lablab* and *Desmodium* spp.) produce small non-nitrogen-fixing nodules that do not have greatly defective structures on their roots. This may explain the findings of Borthakur *et. al.* (1986) where a particular polysaccharide synthesis mutant (*pss*) formed normal nitrogen-fixing nodules on *Phaseolus* beans (a determinate nodule-forming plant), but prevented nodulation of peas (an indeterminate

nodule-forming plant) when the mutation was tested in the *R. leguminosarum* background.

Exo$^-$ mutants may be defective in more than just the EPS synthesis. Chemical analysis of spontaneous Exo$^-$ mutant of *R. leguminosarum* (Exo-1) which does not nodulate peas shows that the Exo-1 mutant does not produce significant amounts of either EPS or CPS but produce some new lipopolysaccharide (LPS) fragments which are not produced by the parent strain (Carlson and Lee, 1983). Some Exo$^-$ mutants of strain NGR234 were not intercellularly-complemented or were partially-complemented by an Exo$^+$ Nod$^-$ mutant (Table 4.1). This suggests that some other surface molecules such as LPS or glucans also may be altered in the Exo$^-$ mutants. These altered molecules may elicit plant defence responses at different stages of nodule development and have a dominant effect to the EPS produced by the Nod$^-$ strain.

Specific recognition of *Rhizobium* by plants may lead to the establishment of compatible host-*Rhizobium* (susceptible plant response) or to an incompatible relationship (resistant plant reaction). Plants have evolved a range of highly effective mechanisms against microbial attack. Similarly, *Rhizobium* have evolved a variety of strategies for bypassing or suppressing these defence mechanisms. Work described in this thesis suggests that *Rhizobium* surface polysaccharides are involved in the molecular communication that is essential for the establishment of a nitrogen-fixing symbiosis between the host plant and bacterium. Genes required in polysaccharide production, and regulation together with *Rhizobium* host specific nodulation (*hsn*) genes, are probably involved in the modulation of the plant defence system.

10.4 FUTURE RESEARCH

A number of research guidelines can be formulated from the research presented in this thesis.

1. The use of *Rhizobium* mutants as "probes" to induce the plant defence response.

It is clear from the results of chapters 3 and 4, that blockade of the invasion of mutant strains with altered polysaccharide production occurs at different stages of nodule development. It will be very important to further chemically analyse these mutants and establish which specific components of the total cell surface polysaccharides are lost. A set of these particular mutants then can be used as "probes" to elicit the induction of the plant defence responses against an "irritant" microorganism at different stages of nodule development. By using these specific mutants, it is hoped that bacterial elicitors will be isolated and characterized. Control of the plant's induced defence response may be a key to understanding the maintenance and stability of the infection by rhizobia. This approach also can lead to a fundamental analysis of the general plant resistance mechanisms and could enable us to devise more precise and reliable ways of controlling plant disease.

2. Characterization of oligosaccharide fragments and their precise biological functions.

Although EPS or oligosaccharide repeat-unit can correct the symbiotic defects of Exo^- mutants, the efficiency of such effective nodulation of plants by Exo^- mutants is lower than that by the wild-

type strain (sections 7.2 and 7.3). It will be interesting to see whether host root exudate can modify the bacterial EPS and if it can, whether such host-modified EPS when added to Exo^- mutants can enhance the effective nodulation ability of the Exo^- mutants. Future experiments should also attempt to determine which parts of the acidic EPS are required for the restoration of a Fix^+ phenotype with Exo^- mutants. Although the addition of the nonasaccharide repeat-unit is sufficient for the restoration of a Fix^+ phenotype, smaller oligosaccharide fragments may be sufficient to act as a signal molecule. By using the spot-inoculation technique (Bhuvaneswari *et. al.*, 1980), it will be possible: (a) to locate the region on plant roots where the oligosaccharide fragments act and; (b) to test whether these fragments induce host gene expression.

3. Sequence analysis of *exo* loci A, E and F.

Nucleotide sequence analysis of two dominant loci E and F will generate some insight into the types of gene products they may encode. Sequence data will also allow the precise determination of Tn*5* insertion sites for some of the mutant alleles at these loci, and hence verify the nature of their mutant gene products (*i.e.* truncated or fused). Nucleotide sequence analysis of *exo* locus A will answer whether it is a promoter site(s). These, in turn, may facilitate a manipulation of this system in order to induce changes in the EPS. Different backbone structures or chemical substitutions may induce altered biological activity. Such manipulations may also explain how the acidic EPS influences the broad-host-range characteristic of strain NGR234.

Fig. 10.1 Diagramatic summary of microscopic stuies done on indeterminate nodules formed on clovers, peas, alfalfa and *Leucaena* after inoculation with wild-type *Rhizobium* strains and mutants defective in polysaccharide synthesis.

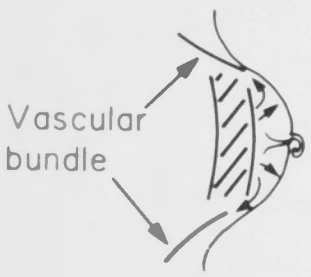
NODULE INDUCTION

INDETERMINATE NODULES

PARENT STRAIN

Invasion via infection thread
(Inf)

MUTANT STRAINS
(Muc⁻)

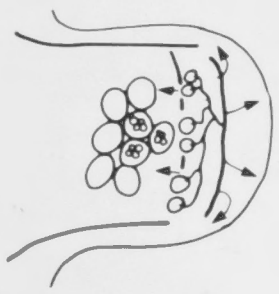


Cortical cells induced into meristematic growth

Less meristematic cell growth

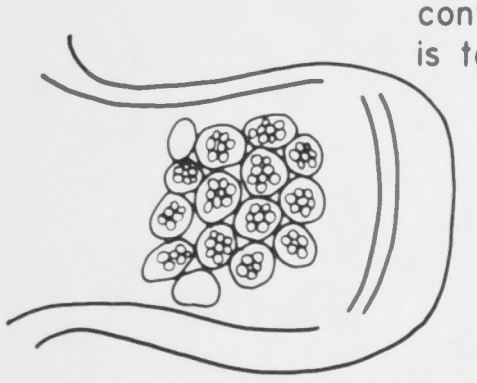
Nodule initiation ahead of advancing infection thread
(Noi)

Less ramification of infection thread



Development of branched infection thread and invasion of dividing meristematic cells.
(Inb)

Induced cell division must be maintained and continued if a nodule is to form
(Nod)



Less bacteroid development and early bacteroid breakdown

LITERATURE CITED

Abe, M., J.E. Sherwood, R.I. Hollingsworth and F.B. Dazzo. (1984) Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. J. Bacteriol. 160: 517-520.

Agarwal, A.K., and D.L. Keister. (1983) Physiology of ex planta nitrogenase activity in *Rhizobium japonicum*. Appl. Environ. Microbiol. 45: 1592-1601.

Albersheim, P., and A.J. Anderson Prouty. (1975) Carbohydrates, proteins, cell surface, and the biochemistry of pathogenesis. Annu. Rev. Plant Physiol. 26: 31-52.

Akkermans, A.D.L., S. Abdulkadir and M.J. Trinick. (1978) N_2-Fixing root nodules in Ulmaceae: *Parasponia* or (and) *Trema* spp.? Plant and Soil 49: 711-715.

Allen, E.K. and O.N. Allen. (1981) The leguminosae: A source book of characteristics, used and nodulation. Macmillan Publishing Ltd., London.

Appelbaum, E.R., E. Johansen and N. Chartrain. (1985) Symbiotic mutants of USDA191, a fast-growing *Rhizobium* that nodulates soybeans. Mol. Gen. Genet. 201: 454-461.

Appelby, C.A., J.D. Tjepkema and M.J. Trinick. (1983) Hemoglobin in a nonleguminous plant, *Parasponia*: possible genetic origin and function in nitrogen fixation. Science. 220: 951-953.

Bachem, C.W.B., Z. Banfalvi, E. Kondorosi, J. Schell and A. Kondorosi. (1986) Identification of host range determinants in the *Rhizobium* species MPIK 3030. Mol Gen. Genet. 203: 42-48.

Bachem, C.W.B., E. Kondorosi, Z. Banfalvi, A. Kondorosi and J. schell. (1985) Identification and cloning of nodulation genes from the wide host range *Rhizobium* strain MPIK 3030. Mol. Gen. Genet. 199: 271-287.

Bagdasarian, M., R. Lurz, B. Rückert, F.C.H. Franklin, M.M. Bagdasarian, J. Frey and K.N. Timmis. (1981) Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 16: 237-247.

Balassa, R. (1956) Durch desoxyribonucleinsauren induzierto veranderungen und *Rhizobien*. Naturwiss. 43: 965-971.

Balassa, R. (1960) Transformation of a strain of *Rhizobium lupini*. Nature 188: 246-247.

Banfalvi. Z., G.S. Randhawa, E. Kondorosi, A. Kiss, and A. Kondorosi. (1983) Construction and characterization of R-prime plasmids carrying symbiotic genes of *R. meliloti*. Mol. Gen. Genet. 189:129-135

Bassett, B., R.N. Goodman and A. Novacky. (1977a) Ultrastructure of soybean nodules. I. Release of rhizobia from the ifection thread. Can. J. Microbiol. 23: 573-582.

Bassett, B., R.N. Goodman and A. Novacky. (1977b) Ultrastructure of soybean nodules. II: deterioration of symbiosis in ineffective nodules. Can. J. Microbiol. 23: 873-883.

Bauer, W.D. (1981) The infection of legumes by rhizobia. Ann. Rev. Plant Physiol. 32: 407-449.

Bauer, W.D., T.V. Bhuvaneswari, A.T. Mort and G. Turgeon. (1979) The initiation of infections in soybean by *Rhizobium*. 3. *R. japonicum* polysaccharide pretreatment induces root hair infectibility. Plant Physiol. 63 (suppl) 135 pp.

Becking, J.H. (1977) Dinitrogen fixing associations in higher plants other than legumes. In: R.W.F. Hardy and W.S. Silver (eds.): A Treatise in Dinitrogen Fixation, Section III, John Wiley and Son, New York. pp 185-275.

Becking, J.H. (1979) Root nodule symbiosis between *Rhizobium* and *Parasponia* (Ulmacea). Plant Soil. 51: 289-296.

Becking, J.H. (1983) The *Parasponia parviflora-Rhizobium* symbiosis. Host specificity, growth and nitrogen fixation under various conditions. Plant and soil 75: 309-342.

Beijerinck, M.W. (1888) Die bacterien der Papilionaceen knollchen. Bot. Ztg. 46: 726-804

Bender, G.L., W. Goydych, B.G. Rolfe and M. Nayudu. (1987a) The role of *Rhizobium* conserved and host specific nodulation genes in the infection of the non-legume *parasponia andersonii*. Mol. Gen. Genet. (in press).

Bender, G. L., M. Nayudu and B. G. Rolfe. (1987b) Early infection events in the nodulation of the non-legume *Parasponia andersonii* by *Bradyrhizobium*. Plant Sci. (in press).

Bender, G. L., and B. G. Rolfe. (1985) A rapid plant assay for the *Parasponia-Rhizobium* symbiosis. Plant Sci. 38: 135-140.

Berg, D.E., and C.M. Berg. (1983) The prokaryotic transposable element Tn*5*. Biotechnology 1: 417-435.

Berg, D.E., A. Weiss and L. Crossland. (1980) Polarity of Tn*5*-insertion mutations in *Escherichia coli*. J. Bacteriol. 142: 439-446.

Bergersen, F.J. (1961) The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. 14: 349-360.

Bergersen, F.J., and G.L. Turner. (1967) Nitrogen fixation by bacterial fraction of breis of soybean root nodules. Biochem, Biophys. Acta. 141: 507-515.

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84: 188-498.

Beringer, J.E., J.L. Beynon, A.V. Buchanan-Wollaston and A.W.B. Johnston. (1978a) Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature 276: 633-634.

Beringer, J.E., N.J. Brewin and A.W.B. Johnston. (1980) The genetic analysis of *Rhizobium* in relation to symbiotic nitrogen fixation. Heredity 45: 161-186.

Beringer, J.E., S.A. Hoggan and A.W.B. Hohnston. (1978b) Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. Gen. Microbiol. 104: 201-207.

Beringer, J.E., and D.A. Hopwood. (1976) Chromosomal recombination and mapping in *Rhizobium leguminosarum*. Nature 264: 291-293.

Berry, A.M., and J.G. Torrey. (1983) Root hair deformation in the infection process of *Alnus rubra*. Can. J. Bot. 61: 2863-2876.

Bhagwat, A.A., and J. Thomas. (1984) Legume-*Rhizobium* interactions: host induced alterations in capsular polysaccharides and infectivity of cowpea rhizobia. Arch. Microbiol. 140: 260-264.

Bhagwat, A.A., and J. Thomas. (1987) *Rhizobium* induced plant proteins in target root epidermal cells of *Vigna unguiculata*. In: D.P.S. Verma and N. Brisson (eds.): Molecular Biology in Plant-Microbe Interactions, Current Plant Science and Biotechnology in Agriculture, Marthinus Nijhoff Publishers. pp 120-122.

Bhuvaneswari, T.V., A.A. Bhagwat and W.D. Bauer. (1981) Transient susceptibitity of root cells in four common legumes to nodulation by rhizobia. Plant Physiol. 68: 1144-1149.

Bhuvaneswari, T.V., B.G. Turgeon and W.D. Bauer. (1980) Early events in the infection of soybean (*Glycinemax* Merr) by *Rhizobium japonicum*. I. Localization of infectible root cells. Plant Physiol. 66: 1027-1031.

Birnboim, H.C., and J. Doly. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1512-1523.

Bitter, T., and H. M. Muir. (1962) A modified uronic acid carbazole reaction. Analy. Biochem. 4: 330-334.

Bohlool, B.B., and E.L. Schmidt. (1974) Lectins: A possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185: 269-271.

Bolivar, F., R.L. Rodriguez, P.L. Greene, M.C. Betlach, H.L. Heyneker, H.W. Boyer, J.H. Crosa and S. Falkow. (1977) Construction and characterization of new cloning vehicles II. A multipurpose cloning system. Gene 2: 95-113.

Bolton, E., P. Glynn and F. O'gara. (1984) Site specific transposition of Tn7 into a *Rhizobium meliloti* megaplasmid. Mol. Gen. Genet. 193: 153-157.

Bond, G. (1974) Root nodule symbiosis with actinomycete-like organisms. In: A. Quispel (ed.): The Biology of Nitrogen Fixation, North Holland Publ. Co., Amsterdam. pp. 342-380.

Borthakur, D., C.E. Barber, J.W. Lamb, J.A. Downie and A.W.B. Johnston. (1986) A mutation that blocks exopolysaccharide synthesis prevents nodulation of peas by *Rhizobium leguminosarum* but not of beans by *R. phaseoli* and is corrected by cloned DNA from *Rhizobium* or the phytopathogen *Xanthomonas*. Mol. Gen. Genet. 203: 320-323.

Borthakur, D., J.A. Bownie, A.W.B. Johnston and J.W. Lamb. (1985) *psi*, a plasmid-linked *Rhizobium phaseoli* gene that inhibits exopolyasccharide production and which is required for symbiotic nitrogen fixation. Mol. Gen. Genet. 200: 278-282.

Borthakur, D., and A.W.B. Johnston. (1987) Sequence of *psi*, a gene on the symbiotic plasmid of *Rhizobium phaseoli* which inhibits exopolysaccharide synthesis and nodulation and demonstration that its transcription is inhibited by *psr*, another gene on the symbiotic plasmid. Mol. Gen. Genet. 207: 149-154.

Boucher, C., B. Bergeron, M. Barate DeBertalmio and J. Denarie. (1977) Introduction of bacteriophage Mu into *Pseudomonas solanacearum* and *Rhizobium meliloti* using the R factor RP4. J. Gen. Microbiol. 98: 253-263.

Brewin, N.J., J.E. Beringer and A.W.B. Johnston. (1980) Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120: 413-420.

Caldwell, B.E., and H.G. Vest. (1977) Genetic aspects of nodulation and dinitrogen fixation by legumes: The macrosymbiont. In: R.W.F. Hardy and W.S. Silver (eds.): A Treatise on Dinitrogen Fixation, section III, London, John Wiley and Son, New York. pp 557-575.

Canter Cremers, H.C.J., A.A.N. van Brussel, J. Plazinski and B.G. Rolfe. (1986) Sym plasmid and chromosomal gene products of *Rhizobium trifolii* elicit developmental responses on various legume roots. J. Plant Physiol. 122: 25-40.

Carlson, R.W. (1982) *Rhizobium* Sruface chemistry. In: W.J. Broughton (ed.): Nitrogen fixation Vol. 2: *Rhizobium*, Oxford University Press, London. pp 199-234.

Carlson, R.W., F. Hustmeyer, B.G. Rolfe and M.A. Djordjevic. (1986) A structural comparison of the acidic extracelluar polysaccharides from *Rhizobium trifolii* mutants affected in root hair infection. Plant Physiol. 80: 134-137.

Carlson, S.W., and R.P. Lee. (1983) A comparison of the surface polysaccharides from *Rhizobium leguminosarum* 128C53 $sm^r rif^r$ with the surface polysaccharides from its Exo-1 mutant. Plant Physiol. 71: 223-228.

Carlson, R.W., and M. Yadav. (1985) Isolation and partial characterization of the extracellular polysaccharides and lipopolysaccharides from fast-growing *Rhizobium japonicum* USDA205 and its Nod^- mutant, HC205, which lacks the symbiotic plasmid. Appl. Environ. Microbiol. 50: 1219-1224.

Casadesus, J., E. Ianez and J. Olivares. (1980) Transposition of Tn*1* to the *Rhizobium meliloti* genome. Mol. Gen. Genet. 180: 405-410.

Chakravorty, A.K., W. Zurkowski, J. Shine and B.G. Rolfe. (1982) Symbiotic nitrogen fixation: Molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J. Mol. Appl. Genet. 1: 2585-596.

Chandler, M.R. (1978) Some observations of *Arachis hypogaea* L. nodulation by *Rhizobium*. J. Exp. Bot. 29: 749-755.

Chandler, M.R., R.A. Date and R.J. Roughley. (1982) Infection and root nodule development in *Stylosanthes* species by *Rhizobium*. J. Exp. Bot. 33: 47-57.

Chang, A.C.Y., and S.N. Cohen. (1978) Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134: 1141-1156.

Chen, A.P.T., and D.A. Philips. (1976) Attachment of *Rhizobium* to legume roots as the basis for specific interaction. Physiol. Plant 38: 83-88.

Chen. H., M, Batley, J.W. Redmond, and B.G. Rolfe. (1985) Alteration of the effective nodulation properties of a fast-growing broad-host range *Rhizobium* due to changes in exopolysaccharide synthesis. J. Plant Physiol. 120: 331-349.

Chen. H., and B.G. Rolfe. (1987) Cooperativity between *Rhizobium* strains: Induction of nitrogen-fixing nodules on the tropical legume *Leucaena leucocephala*. J. Plant Physiol. 127: 307-322.

Cleary, P., A. Campbell and R. Chang. (1972) Location of promoter and operator sites in the biotin gene cluster. Proc. Natl. Acad. Sci. 69: 2219-2223.

Corbin, D., L. Barran and G. Ditta. (1983) Organisation and expression of *R. meliloti* nitrogen fixation genes. Proc. Natl. Acad. Sci. 80: 3005-3009.

Coventry, D.R., M.J. Trinick and C.A. Appelby. (1976) A search for leghaemoglobin-compound in root nodules of *Trema cannabina* Lour. Biochem. Biophys. Acta. 420: 105-111.

Darvill, A.G., and P. Albersheim. (1984) Phytoalexins and their elicitors - a defence against microbial infections in plants. Annu. Rev. plant Physiol. 35: 243-275.

Dazzo, F.B. (1980) Determinants of host specificity in the *Rhizobium*-clover symbiosis. In: W.E. Newton and W.H. Orme-Hohoson (eds.): Nitrogen Fixation, Vol. II, Uni. Park Press (Baltimore). pp 165-187.

Dazzo, F.B., and A.E. Gardiol. (1984) Host specificity in *Rhizobium*-legume interactions. In: D.P.S. Verma and Th. Hohn (eds.): Genes Involved in Microbe-Plant Interactions, Springer-Verlag, Wien. pp 3-31.

Dazzo, F.B., and D.H. Hubbell. (1975) Cross-reactive antigens and lectin as determinants of symbiotic specificity in the *Rhizobium*-clover association. Appl. Microbiol. 30: 1017-1033.

Dazzo, F.B., and D.H. Hubbell. (1976) Concanavalin A: Lack of correlation between binding to *Rhizobium* and specificity in the *Rhizobium*-legume symbiosis. Plant Soil 43: 713-717.

Dazzo, F.B., C. Napoli and D.H. Hubbell (1976) Adsorption of bacteria to roots as related to host specificity in the *Rhizobium*-clover symbiosis. Appl. Environ. Microbiol. 32: 166-171.

Dazzo, F.B., G.L. Truchet, J.E. Sherwood, E.M. Hrabak, and A.E. Gardiol. (1982) Alteration of the trifoliin A-binding capsule of *Rhizobium trifolii* 0403 by enzymes released from clover roots. Appl. Environ. Microbiol. 44: 478-490.

Dart, P.J. (1977) Infection and development of leguminous nodules. In: R.W.F. Hardy and W.S. Silver (eds.): A Treatise on Dinitrogen Fixation. Section III. Biology, John Willey and Sons, New York. pp 367-472.

Datta, N., R.W. Hedges, E.J. Shaw, R.B. Sykes and M.H. Richmod. (1971) Properties of an R factor from *Pseudomonas aeruginosa*. J. Bacteriol. 108: 1244-1249.

deFaria, S.M., J.M. Sutherland and J.I. Sprent. (1986) A new type of infected cell in root nodules of *Audira* spp. (Leguminosae). Plant Sci. 45:143-147.

Dickinson, C.H., and J.A. Lucas. (1982) Plant Pathology and Plant Pathogens. Blackwell Scientific Publications, Oxford, England.

Djordjevic, M.A. (1983) A genetic and molecular characterization of plasmid genes involved in the early nodulation steps of *Rhizobium trifolii*. PhD. Thesis, Australian National University, Canberra.

Djordjevic, M.A., D.W. Gabriel and B.G. Rolfe. (1987) *Rhizobium* the refined parasite of legumes. Ann. Rew. Phytopathology (in press).

Djordjevic, M.A., R.W. Innes, C.A. Wijffelman, P.R. Schofield and B.G. Rolfe. (1986) Nodulation of specific legumes is controlled by several distinct loci in *Rhizobium trifolii*. Plant Mol. Biology. 6: 389-401.

Djordjevic, M.A., P.R. Schofield, R.W. Ridge, N.A. Morrison B.J. Bassam, J. Plazinski, J.M. Watson and B.G. Rolfe. (1985) *Rhizobium* nodulation genes involved in root hair curling (Hac) are functionally conserved. Plant Mol. Biol. 4: 147-160.

Djordjevic, M.A., W. Zurkowski and B.G. Rolfe. (1982) Plasmids and stability of symbiotic properties of *Rhizobium trifolii*. J. Bacteriol. 151: 560-568.

Djordjevic, M.A., W. Zurkowski, J. Shine and B.G. Rolfe. (1983). Sym plasmid transfer to symbiotic mutants of *Rhizobium trifolii*, *Rhizobium leguminosarum* and *Rhizobium meliloti*. J. Bacteriol. 156: 1035-1045.

Djordjevic, S.P. (1987) A chemical and genetic analysis of the role of *Rhizobium* exopolysaccharides in the infection of legume species. PhD thesis, Australian National University, Canberra.

Djordjevic, S.P., H. Chen, M. Batley, J.W. Redmond and B.G. Rolfe. (1987a) Nitrogen fixation ability of exopolysaccharide synthesis mutants of *Rhizobium* sp. strain NGR234 and *Rhizobium trifolii* is restored by the addition of homologous exopolysaccharides. J. Bacteriol. 169: 53-60.

Djordjevic, S.P., H. Chen, J.X. Gray, J.J. Weinman, M.A. Djordjevic, J.W. Redmond, M. Batley and B.G. Rolfe. (1987b) *Rhizobium* exopolysaccharides are essential for the formation of nitrogen-fixing nodules in the *Rhizobium*-legume symbiosis. In: D.P.S. Verma and N. Brisson (eds.): Molecular Biology in Plant-Microbe Interactions, Current Plant Science and Biotechnology in Agriculture, Martinus Nijhoff Publishers. pp 173-178.

Djordjevic, S.P., J. Redmond, M. Batley and B.G. Rolfe. (1986) The structure of the exopolysaccharide from *Rhizobium* sp. strain ANU280 (NGR234). Carbohydrate Res. 148: 87-99.

Doke, N., K. Tomiyama and N. Furuichi. (1982) Elicitation and suppression of the hypersensitive response in host-parasite specificity. In: Y. Asada, W.R. Bushnell, S. Ouchi and C.P. Vance (eds.): Plant infection: the physiological and biochemical basis, Japan Scientific Press, Tokyo, Springer-Verlag, Berlin.

Downie, J.A., C.D. Knight, A.W.B. Johnston and L. Rossen. (1985) Identification of genes and gene products involved in the nodulation of peas by *Rhizobium leguminosarum*. Mol. Gen. Genet. 198: 255-262.

Downie, J.A., Q-S. Ma, C.D. Knight, G. Hombrecher and A.W.B. Johnston. (1983) Cloning of the symbiotic region of *Rhizobium* leguminosarum: the nodulation genes are between the nitrogenase genes and a *nif*A-like gene. EMBO Journal 2: 947-952.

Duhoux, E. (1984) Ontogenese des nodules caulinaires du *Sesbania rostrata* (legumineuses). Can. J. Bot. 62:982-984.

Dunican, L.K., and F. C. Cannon. (1971) The genetic control of symbiotic properties in *Rhizobium*: Evidence for plasmid control. Plant and Soil, Special Volume. pp 73-79.

Eckhardt, T. (1978) A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1: 584-588.

Elligboe, A.H. (1972) Genetics and physiology of primary infection of *Erysiphe graminis*. Phytopathology. 62: 401-406.

Ervin, S.E., and D.H. Hubbell. (1985) Root hair deformations associated with fractionated extracts from *Rhizobium trifolii*. Appl. Environ. Microbiol. 49: 61-68.

Finan, T.M., B. Kunkel, G.F. De Vos and E.R. Signer. (1986) Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. 167: 66-72.

Fisher, R.F., J.K. Tu and S.R. Long. (1985) conserved nodulation genes in *R. meliloti* and *R. trifolii*. Appl. Environ. Microbiol. 39: 1432-1435.

Fred, E.B., I.R. Baldwin and E. McCoy. (1932) Root nodule bacteria and leguminous plants. University of Wisconsin Studies, Science N°5 343 pp.

Gamborg, O. L., and D. Eveleigh. (1968) Detection of gluconases in suspension cultures of barley. Can. J. Biochem. 46: 417-422.

Gibson, A.H. (1963) Physical environment and symbiotic nitrogen fixation. I. The effect of root temperature on recently nodulated *Trifolium subterraneum* L. plants. Aust. J. Biol. Sci. 16: 28-42.

Gnanamanickam, S.S., and S.S. Patil. (1977) Phaseotoxin suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. Physiol. Plant Pathol. 10: 169-179.

Goodchild, D.J., and F.J. Bergersen. (1966) Electron microscopy of the infection and subsequent development of soybean nodule cells. J. Bacteriol. 92: 204-213.

Gottesman, S., P. Trisler and A. Torres-Cabassa. (1985) Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: Charaterization of three regulatory gene. J. Bacteriol. 162: 1111-1119.

Gresshoff, P.M., and B.G. Rolfe. (1978) Viability of *Rhizobium* bacteroid isolated from soybean nodule protoplasts. Planta 142: 329-333.

Gresshoff, P.M., M.L. Skotnicki, J.F. Eadie and B.G. Rolfe. (1977) Viability of *Rhizobium trifolii* bacteroids from clover root nodules. Plant Sci. Lett. 10: 299-304.

Guha, A., Y. Saturen and W. Szybalski. (1971) Divergent orientation of transcription from the biotin locus of *Escherichia coli*. J. Mol. Biol. 56: 53-62.

Haas, D., and B.W. Holloway. (1976) R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. 144: 243-251.

Hamblin, J., and S.P. Kent. (1973) Possible role of phytoheamagglutination in *Phaseolus vulgaris* L. Nature 245: 28-30.

Heath, M.C. (1974) Light and electron microscope studies of the interactions of host and non-host plants with the cowpea rust, *Uromyces phaseol* var. vignae. Physiol. Plant Pathol. 4: 403-414.

Heumann, W. (1968) Conjugation in star-forming *Rhizobium lupini*. Mol. Gen. Genet. 102: 132-144.

Higashi, S. (1967) Transfer of white clover infectivity of *Rhizobium trifolii* to *Rhizobium phaseoli* as mediated by an episomic factor. J. Gen. Appl. Microbiol. 13: 391-403.

Higashi, S., and M. Abe. (1980) Promotion of infection thread formation by substances from *Rhizobium*. Appl. Environ. Microbiol. 39: 297-301.

Holloway, B.W. (1979) Plasmids that mobilise bacterial chromosomes. Plasmid 2: 1-19.

Holmes, D.S. and M. Quigley. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197.

Hombrecher, G., R. Gotz, N.J. Dibb, J.A. Downie, A.W.B. Johnston and J. Brewin. (1984) Cloning and mutagenesis of nodulation genes from *Rhizobium leguminosarum* TOM, a strain with extended host range. Mol. Gen. Genet. 194: 283-298.

Horvath, B., E. Kondorosi, M. John, J. Schmidt, I. Torok, Z. Gyorpypal, I. Barabas, U. Wieneke, J. Schell and A. Kondorosi. (1986) Organisation, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell 46: 335-343.

Hubbell, D.H. (1970) Studies on the root hair "curling factor" of *Rhizobium*. Bot. Gaz. 131: 337-342.

Hynes, M.F., R. Simon, P. Müller, K. Niehaus, M. Labes, and A. Püler. (1986) The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. 202: 356-362.

Ingram, D.S., J.A. Sargent and I.C. Tommerup. (1976) Structural aspects of infection by biotrophic fungi. In: J. Friend and D.R. Threlfall (eds.): Biochemical aspects of plant-parasite relationships, Academic Press.

Jarman, T.R., and G.W. Pace. (1984) Energy requirements for microbial exopolysaccharide synthesis. Arch. Microbiol. 137: 231-235.

Johnston, A.W.B., J.L. Beynon, A.V. Buchanan-Wollaston, S.M. Setchell, P.R. Hirsch and J.E. Beringer. (1978) High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature 276: 634-636.

Jordan, D.C. (1984) Section 4. Gram-negative aerobic rods and cocci: Family III. *Rhizobiaceae*: Genus II. *Bradyrhizobium*. In: Williams and Wilkins (eds.): Bergey's Manual of Systematic Bacteriology, vol. 1, Baltimore. pp 234-256.

Jordan, D.C., I. Grinyer and W.H. Coulter. (1963) Electron microscopy of infection threads and bacteria in young root nodules on *Medicago sativa*. J. Bacteriol. 86: 125-137.

Jorgensen, R.A., S.J. Rothstein and W.S. Reznikoff. (1979) A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. genet. 177: 65-72.

Kahn, M.L., J.D. Krans and J.E. Somerville. (1985) A model of nutrient exchange in the *Rhizobium*-legume symbiosis. In: H.J. Evans, P.J. Bottomley and W.E. Newton (eds.): Nitrogen Fixation Research Progress, Martius Nijhoff, Dordrecht. pp 193-199.

Kato, G., Y. Maruyama and M. Nakamura. (1980) Role of bacterial polysaccharides in the adsorption process of the *Rhizobium*-pea symbiosis. Agric. Biol. Chem. 44: 2843-2855.

Keim, T.T.V., P. Toubart, A Lousson, A.G. Darvill, D.J. Gollin, P Chelf, and P. Albersheim. (1985) Manipulation of the morphogenetic pathway of tobacco explants by oligosaccharins. Nature 314: 615-617.

Ketner, G., and A. Campbell. (1975) Operator and promoter mutations affecting divergent transcription in the *bio* gene cluster of *Escherichia coli*. J. Mol. Biol. 96: 13-27.

Kleckner, N. (1981) Translocatable elements in prokaryotes. Annu. Rev. Genet. 15: 341-404.

Klein, S., A.M. Hirsch, C.A. Smith and E.R. Signer. (1987) Coinoculation with symbiotically defective mutants of *Rhizobium meliloti*. In: D.P.S. Verma and N. Brisson (eds.): Molecular Biology in Plant-Microbe Interactions, Current Plant Science and Biotechnology in Agriculture, Martinus Nijhoff Publishers. pp 179-181.

Klement, Z. (1982) Hypersensitivity. In: M.S. Mount and G.H. Lacy (eds.): Phytopathogenic Prokaryotes, Academic Press.

Knight, C.D., L. Rossen, J.G. Robertson, B. Wells and J.A. Downie. (1986) Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nod*ABC genes and analysis of early stages of plant infection. J. Bacteriol. 166: 552-558.

Kondorosi, E., Z. Banfalvi and E. Kondorosi. (1984) Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: Identification of nodulation genes. Mol. Gen. Genet. 193: 445-452.

Kondorosi, A., B. Horvath, K. Rostas, M. Göttfert, P. Putnoky, F. Rodriguez-Quinones, Z. Banfalvi and E. Kondorosi. (1986) Common and host-nodulation genes of *Rhizobium meliloti*. In: D.P.S. Verma (ed.): Molecular Genetics of Plant-Microbe Interactions, Montreal. 50 pp.

Kondorosi, A., G.B. Kiss, T. Forrai, E. Vincze and Z. Banfalvi. (1977) Circular linkage map of *Rhizobium meliloti* chromosome. Nature 268: 525-527.

Kondorosi, A., E. Kondorosi, C.E. Pankhurst, W.J. Broughton and Z. Banfalvi. (1982) Mobilisation of a *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes into other rhizobia and *Agrobacterium*. Mol. Gen. Genet. 188: 433-439.

Kondorosi, A., E. Vincze, A.W.B. Johnston and J.E. Beringer. (1980) A comparison of three *Rhizobium* linkage maps. Mol. Gen. Genet. 178: 403-408.

Lalonde, M. (1977) Infection process of the Alnus root nodule symbiosis. In: W. Newton, J.R. Postgate and C. Rodriguez-Barrueco (eds.): Recent Developments in Nitrogen Fixation, Academic Press, London. 569 pp.

Lamb, J.W., and H. Hennecke. (1986) In *Bradyrhizobium japonicum* the common nodulation genes, *nodABC*, are linked to *nifA* and *fixA*,. Mol. Gen. Genet. 202: 512-517.

Lancelle, S.A., and J.G. Torrey. (1984) Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. I. Infection and early nodule initiation. Protoplasma 123:26-37.

Lancelle, S.A., and J.G. Torrey. (1985) Early development of *Rhizobium*-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. Can. J. Bot. 63:25-35.

Landsmann, J., E.S. Dennis, T.J.V. Higgins, C.A. Appleby, A.A. Kortt and W.J. Peacock. (1986) Common evolutionary origin of legume and non-legume plant haemoglobins. Nature 324:166-168.

Law, I.J., and B.W. Strijdom. (1977) some observations on plant lectins and *Rhizobium* specificity. Soil Biol. Biochem. 9: 79-84.

Law, I.J., Y. Yamamoto, A.J. Mort and W.D. Bauer. (1982) Nodulation of soybean by *Rhizobium japonicum* mutants with altered capsule synthesis. Planta 154: 100-109.

Leemans, J., R. Villaroel, B. Silva, M.V. Montagu, and J. Schell. (1980) Direct repetition of a 1.2 Md DNA sequence is involved in site-specific recombination by the P1 plasmid R68. Gene 10: 319-328.

Leigh, J.A., J. Reed and G.C. Walkler. (1987) Symbiotic mutants of *Rhizobium meliloti* which produce non-succinylated exopolysaccharide. In: D.P.S. Verma and N. Brisson (eds.): Molecular Biology in Plant-Microbe Interactions, Current Plant Science and Biotichnology in Agriculture, Martinus Nijhoff Publishers. pp 165-166.

Leigh, J.A., E.R. Signer and G.C. Walker. (1985) Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. 82: 6231-6235.

Leigh, J.A., and G.C. Walker. (1986). Symbiotic mutants of *Rhizobium melliloti* altered in size and acylation of secreted polysaccharide. In: D.P.S. Verma (ed.): Third International Symposium on The Molecular Genetics of Plant-Microbe Interactions, McGill University, Montrëal. 100 pp

Libbenga, K.R., and R.J. Bogers. (1974) Root nodule morphogenesis. In: A. Quispel (ed.): The Biology of Nitrogen Fixation, North Holland Publishing Co., Amsterdam. pp 430-437.

Littlefield, L.J. (1973) Histological evidence for diverse mechanisms of resistance to flax rust, *Melampsora lini* (Ehrenb.) Lev. Physiol. Plant Pathol. 3: 241-247.

Lowry, O.H., N.J. Rosebrough, AL. Farr and R.J. Randall. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Ma, O.S., A.W.B. Johnston, G. Hombrecher and J.A. Downie. (1982) Molecular genetics of mutants of *Rhizobium leguminosarum* which fail to fix nitrogen. Mol. Gen. Genet. 187: 166-171.

Markovitz, A. (1977) Genetics and regulation of bacterial capsular polysaccharide biosynthesis and radiation sensitivity. In: I.W. Sutherland (ed.): Surface Carbohydrates of the Prokaryotic Cell, Academic Press, pp. 415-462.

Meade, H.M., S.R. Long, G.B. Ruvkun, S.E. Broown and F.M. Ausurel. (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon *Tn5* mutagenesis. J. Bacteriol. 149: 114-122.

Meade, H.M., and E.R. Signer. (1977) Genetic mapping of *Rhizobium meliloti*. Proc. Nat. Acad. Sci. 74: 2076-2078.

Megias, M., M.A. Caviedes, A.J. Palomares and J. Perez-Silva. (1982) Use of plasmid R68.45 for constructing a circular linkage map of the *Rhizobium trifolii* chromosome. J. Bacteriol. 149: 59-64.

Miller, J.H. (1972) Experiments in molecular genetics. Cold Spring Harbor Symp Quant. Biol.

Miller, S.A., and D.P. Maxwell. (1984) Ultrastructure of susceptible, host resistant, and nonhost resistant interactions of alfalfa with *Phytophthora megasperma*. Can. J. Bot. 62: 117-128.

Moore, A.T., M. Nayudu and B.W. Holloway. (1983) Genetic mapping in *Methylophilus methylotrophus* AS1. J. Gen. Microbiol. 129: 785-799.

Morrison, D.A. (1979) Transformation and preservation of competent bacterial cells by freezing. In: R. Wu (ed.): Methods in Enzymology, Vol. 68, Academic Press, New York. pp 326-331.

Morrison, N.A. (1984) Nodulation and nitrogen fixation in *Rhizobium* strain NGR234. PhD. Thesis, Australian National University, Canberra.

Morrison, N.A., Y.H. Cen, M.J. Trinick, J. Shine and B.G. Rolfe. (1983) Heat curing of a Sym plasmid in a fast-growing *Rhizobium* sp. that is able to nodulate legumes and the non-legume *Parasponia* sp. J. Bacteriol. 153: 527-531.

Mueller, W.C., and C.H. Beckman. (1974) Ultrastructure of the phenol-storing cells in the roots of banana. Physiol. Plant Pathol. 4: 187-190.

Napoli, C., and P. Albersheim. (1980) *Rhizobium leguminosarum* mutants incapable of normal extracelluar polysaccharide production. J. Bacteriol. 141: 1454-1456.

Nayudu, M., and B.G. Rolfe. (1987) Analysis of R-primes demonstrates that genes for broad host range nodulation of *Rhizobium* strain NGR234 are dispersed on the Sym plasmid. Mol. Gen. Genet. 206: 326-336.

Newcomb, W. (1976) A correlated light and electron microscopic study of symbiotic growth and differentiation in *Pisum sativum* root nodules. Can. J. Bot. 54: 2163-2186.

Newcomb, W. (1980) Control of morphogenesis and differentiation of pea root nodule. In: W.E. Newton and W.H. Orme-Johnson (eds.): Nitrogen Fixation Vol. 2, University Park Press, Baltimore. pp 87-102.

Newcomb W., and L. McIntyre. (1981) Development of root nodules on mung bean (*Vigna radiata*): a reinvestigation of endocytosis. Can. J. Bot. 59: 2478-2499.

Norris, D.O. (1956) legumes in the *Rhizobium* symbiosis. Empire J. Exp. Agric. 24: 246-270.

Norris, D.O. (1965) Acid production by *Rhizobium* a unifying concept. Plant and Soil 22: 143-166.

Nuti, M.P., A.A. Lepidi, R.K. Prakash, R.A. Schilperoort and F.C. Cannon. (1979) Evidence for nitrogen fixation (*nif*) genes on indigenous *Rhizobium* plasmids. Nature 282: 533-535.

Nutman, P.S. (1956) The influence of the legume in root nodule symbiosis: a comparative study of host determinants and functions. Biol. Rev. Cambridge Philos. Soc. 31: 109-151.

Nutman, P.S. (1969) Genetics of symbiosis and nitrogen fixation in legumes. Proc. Roy. Soc. B. 172: 417-437.

O'Farrell, P.H., E. Kutter and M. Nakanishi. (1980) A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. 179: 421-435.

Olivares, J., G.J. Bedmar, and E. Martinez-Molina. (1984) Infectivity of *Rhizobium meliloti* as affected by extracellular polysaccharides. J. Appl. Bacteriol. 56: 389-393.

Otsuka, A., and J. Abelson. (1978) The regulatory region of the biotin operon in *Escherichia coli*. Nature 276: 689-694.

Ow, D.W., and F.M. Ausubel. (1983) Regulation of nitrogen metabolism genes by *nif*A gene product in *Klebsiella pneumoniae*. Nature 301: 307-313.

Pankhurst, C.E., W.J. Broughton, C. Bachem, E. Kondorosi and A. Kondorosi. (1983) Identification of nitrogen fixation and nodulation genes on a large plasmid from a broad-host-range *Rhizobium* spp. In: A. Puhler (ed.): Molecular Genetics of the Bacteria Plant Interaction, Springer-Verlag, Berlin, Heidelberg. pp 169-176.

Pankhurst, C.E., and A.S. Craig. (1978) Effect of oxygen concentration, temperature and combined nitrogen on the morphology and nitrogenase activity of *Rhizobium* sp. strain 32HI in agar culture. J. Gen. Microbiol. 106: 207-219.

Patel, J.J., and A.F. Yang. (1981) Light and electron microscopic studies of nodule structure of alfalfa. Can. J. Microbiol. 27: 36-43.

Plazinski, J., Y.H. Cen and B.G. Rolfe. (1985) General method for the identification of plasmid species in fast-growing soil microorganisms. Appl. Environ. Microbiol. 48: 1001-1003.

Prakash, R.K., R.K. Schilperoort and M.P. Nuti. (1981) Large plasmids of fast-growing rhizobia: Homology studies and location of structural nitrogen fixation (*nif*) genes. J. Bacterial. 145: 1129-1136.

Price, G.D., S.S. Mohapatra and P.M. Gresshoff. (1985) Structure of nodules formed by *Rhizobium* strain ANU289 in the non-legume *Parasponia* and the legume siratro (*Macroptilium atropurpureum*). Bot. Gaz. 145: 444-451.

Pueppke, S.G. (1984) Adsorption of slow- and fast-growing rhizobia to soybean and cowpea roots. Plant Physiol. 75: 924-928.

Puhler, A., M.F. Hynes, D. Kapp, P. Muller and K. Niehans. (1986) Infection mutants of *Rhizobium meliloti* are altered in the acidic exopolysaccharide production. In: B. Lugtenberg (ed.): Recognition in Microbe-Plant Symbioric and Pathogenic Interactions. Nato ASI Series Vol. H4, Springer-Verlag, Berlin, Heidelberg. pp 29-37.

Pull, S.P., S.G. Pueppke, T. Hynowitz and J.H. Orf. (1978) Soybean lines lacking the 120,000 daltons seed lectin. Science 200: 1277-1279.

Reed, J., S. Long, D. Doherty, and G.C. Walker. (1986) Genetic and regulatory studies of exopolysaccharide production in *Rhizobium meliloti*. In: D.P.S. Verma (ed.): Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions, McGill University, Montrëal. 98 pp.

Ridge, R.W. (1985) Infection of the tropical legume *Macroptilium atropurpureum* by the broad-host-range *Rhizobium* strain NGR234. Ph.D. thesis, Australian National University, Canberra.

Ridge, R.W., and B.G. Rolfe. (1986) Sequence of events during the infection of the tropical legume *Macroptilium atropurpureum* Urb. by the broad-host-range, fast-growing ANU240. J. Plant Physiol. 122: 121-137.

Riess. G., B.W. Holloway and A. Pühler. (1980) R68.45, a plasmid with chromosome mobilizing ability (Cma) carries a tandem duplication. Genet. Res. Camb. 36: 99-109.

Robertson, J.G., and K.J.F. Farnden. (1980) The ultrastructure and metabolism of the developing legume root nodule. In: B.J. Miflin, P.K. Stumpf and E.E. Conn (eds.): Amino Acids and Derivatives, Vol. 5: Biochemistry of Plants, A Comprehensive Treatise, New York, Academic Press.

Robertson, J.G., and P. Lyttleton. (1982) Coated and smooth vesicles in the biogenesis of cell walls plasma membranes infection threads and peribacteroid membranes in root hairs and nodules of white clovers. J. Cell Sci. 58:63-78.

Robertson, J.G., P. Lyttleton, S. Bullivant and G.F. Grayston. (1978) Membranes in lupin root nodules. I. The role of golgi bodies in the biogenesis of infection treads and peribacteroid membranes. J. Cell Sci. 30: 129-149.

Roebuck, P., R. Sexton, and J. W. Mansfield. (1978) Ultrastructural observations on the development of the hypersensitive reaction in leaves of *Phaseolus vulgaris* cv. Red Mexican inoculated with *Pseudomonas phaseolicola* (race 1). Physiol. Plant Pathol. 12: 151-157.

Rolfe, B.G., M.A. Djordjevic, N.A. Morrison, J. Plazinski, G.L. Bender, R. Ridge, W. Zurkowski, J.T. Tellam, P.M. Gresshoff and J. Shine. (1983) Genetic analysis of the symbiotic regions in *Rhizobium trifolii* and *Rhizobium parasponia*. In: A. Pühler (ed.): Molecular Genetics of the Bacteria-Plant Interactions, Springer-Verlag Berlin Heidelberg. pp 188-203.

Rolfe, B.G., M.A. Djordjevic, K.F. Scott, J.E. Hughes, J. Badenoch-Jones, P.M. Gresshoff, Y. Cen, W.F. Dudman, W. Zurkowski and J. Shine. (1981a) Analysis of the nodule-forming ability of fast-growing *Rhizobium* strains. In: A.H. Gibson and W.E. Newton (eds.): Current Perspectives in Nitrogen Fixation, Australian Academy of Science, Canberra. pp 142-145.

Rolfe, B.G., and P.M. Gresshoff. (1980) *Rhizobium trifolii* mutant interaction during establishment of nodulation in while clover. Aust. J. Biol. Sci. 33: 491-504.

Rolfe, B. G., P. M. Gresshoff and J. Shine. (1980a) Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci. Letters. 19: 277-284.

Rolfe, B.G., P.M. Gresshoff, J. Shine and J.M. vincent. (1980b) Interaction between a non-nodulating and an ineffective mutant of *Rhizobium trifolii* resulting in effective (nitrogen-fixing) nodulation. Appl. Enr. Microbiol. 39: 449-452.

Rolfe, B.G., J.W. Redmond, M. Batley, H. Chen, S.P. Djordjevic, R.W. Ridge, B.J. Bassam, C.L. Sargent, F.B. Dazzo and M.A. Djordjevic. (1986) Intercellular communication and recognition in the *Rhizobium*-legume symbiosis. In: B. Lugtenberg (ed.): Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions, NATO ASI Series, Vol. H4, Springer-Verlag, Berlin, Heigelberg. pp 39-54.

Rolfe, B.G., and J. Shine. (1984) *Rhizobium*-leguminosae symbiosis: The bacterial point of view. In: D.P.S. Verma and T.H. Hohn (eds.): Plant Gene Research: Genes Involved in Microbe-Plant Interactions, Springer-Verlag Wien, New York. pp 95-128.

Rolfe, B.G., J. Shine, P.M. Gresshoff, K.F. Scott, M. Djordjevic, Y. Cen, J.E. Hughes, G.L. Bender, A.K. Chakravorty, W. Zurkowski, J.M. Watson, J. Badenoch-Jones, N.A. Morrison and M.T. Trinick. (1981b) Transposon induced mutants of fast and slow-growing *Rhizobia* and molecular cloning of symbiotic genes. Proc. 8th North American *Rhizobium* conference, University of Namitoba, Winnipeg, pp 34-54.

Rolfe, B.G., J. Shine, P.M. Gresshoff, K.F. Scott, M.A. Djorjevic, Y. Cen, J.E. Hughes, G.L. Bender, A.K. Chakravorty, W. Zurkowski, J.M. Watson, J. Badenoch-Jones, N.A. Morrison and M.J. Trinick. (1982) *Rhizobium* and the genetics of a controlled disease. Australian Microbiologist 3: 33-37.

Russell, P., G.M. Schell, K.K. Nelson, L.J. Halverson, K.M. Sirotkin and G. Stacey. (1985) Isolation and characterization of the DNA region encoding nodulation functions in *Bradyrhizobium japonicum*. J. Bacteriol. 164: 1301-1308.

Sahlman, K., and G. Fåhraeus. (1963) An electron microscope study of root-fair infection by *Rhizobium*. J. Gen. Microbiol. 33: 425-431.

Sanders, R.E., R.W. Carlson and P. Albersheim. (1978) A *Rhizobium* mutant incapable of nodulation and normal polysaccharide secretion. Nature 271: 240-242.

Sanders, R., E. Raleigh and E. Singer. (1981) Lack of correlation between extracellular polysaccharide and nodulation ability in *Rhizobium*. Nature 292: 148-149.

Schofield, P.R., M.A. Djordjevic, B.G. Rolfe, J. Shine and J.M. Watson. (1983) A molecular linkage map of nitrogenase and nodulation genes in *Rhizobium trifolii*. Mol. Gen. Genet. 192: 459-465.

Schofield, P.R., R.W. Ridge, B.G. Rolfe, J. Shine and J.M. watson. (1984) Host-specific nodulation is encoded on a 14kb DNA fragment in *Rhizobium trifolii*. Plant Mol. Biol. 33: 3-11.

Scott, K.F. (1986) Conserved nodulation genes from the non-legume symbiont *Bradyrhizobium* sp. (*parasponia*). Nucleic Acids Res. 14: 2905-2919.

Scott, K.F., J.E. Hughes, P.M. Gresshoff, J.E. Beringer, B.G. Rolfe and J. Shine. (1982) Molecular cloning of *Rhizobium trifolii* genes involved in symbiotic nitrogen fixation. J. Mol. Appl. genet. 1: 315-326.

Seegers, R., and T.A. LaRue. (1985) Legume agglutinins that bind to *Rhizobium meliloti*. J. Bacteriol. 162: 784-789.

Sequeira, L., G. Gaard and G.A. De Zoeten. (1977) Interaction of bacteria and host cell walls: its relation to mechanisms of induced resistance. Physiol. Plant Pathol. 10: 43-50.

Shields, R., and W. Burnett. (1960) Determination of protein-bound carbohydrate in serum by a modified anthrone method. Analytic Chemistry 32: 885-886.

Simon, R., U. priefer and A. Puhler. (1983) Vector plasmids for *in-vivo* and *in-vitro* manipulations of Gram-negative bacteria. In: A. Puhler (ed.): Molecular Genetics of the Bacteria-Plant Interactions, Springer-Verlag, Berlin. pp 98-106.

Simon, R., G. Weber, W. Arnold and A. Puhler. (1982) Analysis of plasmid borne genes in *Rhizobium meliloti* by Tn*5* mutagenesis. In: K.W. Clarke and J.H.G. Stephens (eds.): Proceedings 8th Nth American *Rhizobium* Conference, University of Manitoba Press. pp 67-89.

Skotnicki, M.L., and B.G. Rolfe. (1979) Bacterial and cateroid properties of mutants of *Rhizobium trifolii* strain T1 uncoupled in oxidative phosphorylation. Aust. J. Biol. Sci. 32: 501-517.

Solheim, B., and K.E. Fjellheim. (1984) Rhizobial polysaccharide-degrading enzymes from roots of legumes. Physiol. Plant 62: 11-17.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Sprent, J.I. (1979) The biology of nitrogen-fixing organisms. Maidenhead, England: McGraw-Hill Ltd.

Stacey, G., A.S. Paau and W.J. Brill. (1980) Host recognition in the *Rhizobium*-soybean symbiosis. Plant Physiol. 66: 609-614.

Stoess, A. (1980) Phytoalexin-biogenic perspective. Phytopath. Z. 99: 251-272.

Sundaresan, V., D.W. Ow and F.M. Ausubel. (1983) Activation of *Klebsiella pneumoniae* and *Rhizobium meliloti* nitrogenase promoters by *gln(ntr)* regulatory proteins. Proc. Natl. Acad. Sci. 80: 4030-4034.

Sutherland, I.W. (1979) Microbial exopolysaccharides: control of synthesis and acylation. In: R.C.W. Berkeley (ed.): Microbial Polysaccharides and Polysaccharases, Academic Press, London. pp 1-35.

Tarr, S.A.J. (1972) Principles of Plant Pathology. MacMillan Press.

Tomiyama, K. (1982) Hypersensitive cell death: its significance and physiology. In: Y. Asada, W.R. Bushnell, S. Ouchi and C.P. Vance (eds.): Plant Infection: The Physiological and Biochemical Basis, Japan Scientific Press, Tokyo, Springer-Verlag Berlin.

Trinick, M.J. (1965) Investigations into the rhizobia of tropical legumes with particular reference to *Leucaena leucocephala* (Lam) De Witt. B. Sc. Agr. Shesis, Sydney University, Sydney, Australia.

Trinick, M.J. (1973) Symbiosis between *Rhizobium* and the non-legume *Trema aspera*. Nature 144: 459-460.

Trinick, M.J. (1979) Structure of nitrogen-fixing nodules formed by *Rhizobium* on roots of *Parasponia andersonii* Plants. Can. J. Microbiol. 25: 565-578.

Trinick, M.J. (1980a) Growth of *Parasponia* in agar tube culture and symbiotic effectiveness of isolates from *Parasponia* app. New Phytol. 85: 37-45.

Trinick, M.J. (1980b) Relationship among the fast-growing rhizobia of *Lablab purpureus, Leucaena leucocephala, Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J. Appl. Bacteriol. 49: 39-53.

Trinick, M.J., and J. Galbraith. (1976) Structure of root nodules formed by *Rhizobium* on the non-legume *Trema cannabina* var. *Scabra*. Arch. Microbiol. 108:159-166.

Trinick, M.J. and J. Galbraith. (1980) The *Rhizobium* requirements of the non-legume *Parasponia* in relationship to the cross-inoculation group concept of legumes. New Phytol. 86: 17-26.

Tully, R.E., and M.E. Terry. (1985) Decreased exopolysaccharide synthesis by anaerobic and symbiotic cells of *Bradyrhizobium japonicum*. Plant Physiol. 79: 445-450.

Turgeon, B.G., and W.D. Bauer. (1985) Ultrastructure of infection thread development during the infection of soybean by *Rhizobium japonicum*. Planta 163:328-349.

van Brussel, A.A.N., K. Planque and A. Quispel. (1977) The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. J. Gen. Microbiol. 101: 51-56.

van Vliet, F., B. Silver, M. van Montagu and J. Schell. (1978) Transfer of RP4::Mu plasmid to *Agrobacterium tumefaciens*. Plasmid 1: 446-455.

Vance, D.P. (1983) *Rhizobium* infection and nodulation: a beneficial plant disease? Annu. Rev. Microbiol. 37: 399-424.

Vasse, J.M., and G.J. Truchet. (1984) The *Rhizobium*-legume symbiosis: Observation of root infection by bright-field microscopy after staining with methylene blue. Planta 161: 487-489.

Verma, D.P.S., and K. Nadler. (1984) Legume-*Rhizobium*-symbiosis: host's point of view. In: D.P.S. Verma and T.H. Hohn (eds.): Plant Gene Research: Genes Involved in Microbe-Plant Interactions, Springer-Verlag Wien, New York. pp 57-93.

Vesper, S.J., and W.D. Bauer. (1985) Characterization of *Rhizobium* attachment to soybean roots. Symbiosis 1: 139-162.

Vincent, J.M. (1970) A manual for the practical study of root-nodule bacteria. IBP handbook No. 15, Blackwell Scientific Publications, Oxford.

Vincent, J.M. (1974) Root-nodule symbiosis with *Rhizobium*. In: W.E. Newton and W.H. Oreme-Johnson (eds.): The Biology of Nitrogen Fixation, Vol. 2, University Park Press, Baltimore. pp 103-129.

Vincent, J.M. (1980) Factors controlling the legume-*Rhizobium* symbiosis. In: W.E. Newton and W.H. Orme-Johnson (eds.): Nitrogen Fixation, Vol. 2, University Park Press, Baltimore. pp 103-129.

Walker, G.C., J.A. Leigh, J. Reed, D. Doherty, and S. Long. (1986) Mutations affecting exopolysaccharide production by *Rhizobium meliloti*. In: D.P.S. Verma (ed.): Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions, Mcgill University, Montrëal. 27 pp.

West, C.A. (1981) Fungal elicitors of the phytoalexin response in higher plants. Naturwissenschaften 68: 447-457.

Willetts, N.S., C. Crowther and B.W. Holloway. (1981) The insertion sequence IS*21* of R68.45 and the molecular basis for mobilization of the bacterial chromosome. Plasmid 6: 30-52.

Wilson, J.K. (1944) Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. Soil Sci. 58: 61-69.

Wilson, K.J., V. Anjaiah, P.T.C. Nambiar and F.M. Ausubel. (1987) Isolation and characterization of symbiotic mutants of *Bradyrhizobium* sp. (*Arachis*) strain NC92: mutants with host-specific defects in nodulation and nitrogen fixation. J. Bacteriol. 169: 2177-2186.

Wong, P.P. (1980) Interactions between *Rhizobia* and lectins of lentil, pea, broad been, and jack bean. Plant Physiol. 65: 1049-1052.

Yao, P.Y., and J.M. Vincent. (1969) Host specificity in the root hair curling "factor" of *Rhizobium* spp. Aust. J. Biol. Sci. 22: 413-423.

Yao, P.Y., and J.M. Vincent. (1976) Factors responsible for the curling and branching of clover root hairs by *Rhizobium*. Plant Soil. 45: 1-16.

Zimmerman, J.L., W.W. Szeto and F.M. Ausubel. (1983) Molecular characterization of Tn*5*-induced symbiotic (Fix^-) mutants of *Rhizobium meliloti*. J. Bacteriol. 156: 1025-1034.

Zurkowski, W. (1980) Specific absorption of bacteria to clover root hairs, related to the presence of the plasmid pWZ2 in cells of *Rhizobium trifolii*. Microbiol. 27: 27-32.

Zurkowski, W., and Z. Lorkiewicz. (1978) Effective method for the isolation of non-nodulating mutants of *Rhizobium trifolii*. Genetic Research 32: 311-314.

Zurkowski, W., and Z. Lorkiewicz. (1979) Plasmid mediated control of nodulation in *Rhizobium trifolii*. Arch. Microbiol. 123: 195-201.